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# Understanding the role of *scalloped* in *Drosophila melanogaster*

by

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This Thesis is Dedicated to my Parents

Abstract

The proper development of an organism requires a special class of genes termed selector genes, which are required to coordinate the formation and identity of tissues. The selector gene *scalloped* (*sd*) is expressed in several different tissues. Work done with SD and its homologs suggest that cofactors are required for the proper regulation of downstream genes. Most of the previous research on SD has been restricted to understanding its role in wing development. To determine the role of the SD protein in tissues outside of the wing, a reagent (VGΔACT) that is able to disrupt SD function was created. The expression of this construct reveals that the SD protein is also critical in the development of the appendages, optic lobe and adult compound eye. Furthermore, the use of a second reagent that is able to titrate potential SD cofactors (SDΔ200) shows that the development of each of these tissues requires the SD protein to bind a cofactor. To gain a better understanding of how SD is patterning these tissues, a search for other possible cofactors was initiated. The search led to the discovery that Nervous Finger -1 (Nerfin-1) is able to associate with the SD protein. Nerfin-1 represents a new class of cofactors that is able to interact with TEAD proteins. Furthermore, deletion analysis shows that the Nerfin-1 and Vestigial (VG) cofactors use a similar sequence motif to dock to the same domain on the SD protein. Finally, how SD is able is able to activate different sets of genes in a tissue specific manner was examined. In the wing disc, the VG cofactor is responsible for inducing this target specificity. However, instead of changing the sequence that the SD protein recognizes, the VG protein alters the affinity the SD protein has for DNA. The reduced affinity for DNA is circumvented by the fact that the VG protein promotes the assembly of a complex that contains two SD proteins. These two features would ensure the SD/VG complex could only bind to enhancers that contain two consecutive binding sites, a feature that to date has been found exclusively in wing enhancers.

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List of Abbreviations

| | |
|---|---|
| 14-3-3 | A highly conserved protein-protein interaction domain that binds to phosophoserine residues |
| a.a. | amino acid |
| ACT | *activation domain* |
| ATTS | ABAA/TEC1p/TEF1/SD |
| bp | base pairs |
| ChIP | chromatin immunoprecipitation |
| CNS | central nervous system |
| cTNT | cardiac troponin |
| D/V | dorsal-ventral |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| EIN | EGL-46/INSM-1/Nerfin-1 |
| EGTA | ethylene glycol tetraacetic acid |
| EMSA | electrophoretic mobility shift assay |
| FRE | filamentous response element |
| GAL4 | GAL4 binding domain of yeast |
| GST | Glutathione S-transferase |
| IgG | immunoglobulin G |
| MCAT | contain the canonical 5' CATTCCT 3' sequence |
| NaCl | Sodium Chloride |
| Ni | nickel |
| NMR | nuclear magnetic resonance |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PBT | phosphate buffered saline with triton X |
| PCR | polymerase chain reaction |
| *Pfu* | *Pyrococcus furiosus* |
| PNS | peripheral nervous system |
| PRE | pheromone response element |
| RNA | ribonucleic acid |
| RNAi | interfering RNA |
| SID | Scalloped interaction domain |
| SDS | sodium dodecyl sulphate |
| SRE | serum response element |
| SRF | serum response factor |
| T1 | thoracic segment 1 |
| T2 | thoracic segment 2 |
| T3 | thoracic segment 3 |
| *Taq* | *Thermus aquaticus* |
| TCS | TEC1p consensus sequence |
| TD | transactivating domain |
| TEA | TEC1p/EGL-44/ABAA |
| TEAD | TEA/ATTS DNA binding domain |
| TIF | transcription intermediary factor |
| TF | transcription factor |
| UAS | upstream activation sequence |
| UV | ultraviolet |
| VID | Vestigial interaction domain |
| WW domain | Domain composed of two tryptophans spaced 20 to 22 amino acids apart |
| Zn | Zinc |

List of Genes

| | |
|---|---|
| α-*MHC* | *α-myosin heavy chain* |
| *ato* | *atonal* |
| *CBP* | *c-terminal binding protein* |
| *dIAP1* | *Drosophila inhibitor of apoptosis-1* |
| *Dig2* | *Dig2* |
| *dll* | *distal-less* |
| *dpp* | *decapentaplegic* |
| *egl-44* | *egglaying-44* |
| *exd* | *extradenticle* |
| *eye* | *eyeless* |
| *hth* | *homothorax* |
| *lab* | *labial* |
| *ladybird* | *ladybird* |
| *mad/medea* | *mothers against dpp* |
| *max* | *max* |
| *MEF-1* | *myocyte enhancing factor-1* |
| *MEF-2* | *myocyte enhancing factor-2* |
| *Nerfin-1* | *nervous finger-1* |
| *pan* | *pannier* |
| *PARP* | *nuclear protein poly(ADP-ribose) polymerase* |
| *ptc* | *patched* |
| *ry* | *rosy* |
| *sb* | *stubble* |
| *sd* | *scalloped* |
| *sev* | *sevenless* |
| *SRF* | *Serum response factor* |
| *Ste12* | *Ste12* |
| *Su(H)* | *suppressor of hairless* |
| *tinmain* | *tinman* |
| *TAZ* | *Transcriptional Co-activator with PDZ-binding doamin* |
| *Tec1* | *Tec1* |
| *TEF-1* | *Transcription Enhancer Factor-1* |
| *vg* | *vestigial* |
| *vgl* | *vestigial-like* |
| *w* | *white* |
| *wg* | *wingless* |
| *YAP65* | *Yes-Associated Protein 65* |
| *yorkie* | *yorkie* |

## CHAPTER I - GENERAL INTRODUCTION

How a zygote is able to go from a single cell to a complex multi-cellular organism has long fascinated scientists. Work directed at how embryos "self-assemble" has revealed that this process requires exquisite control and coordination of many different events from cell division to cell differentiation. The employment of transcription factors, proteins that are able to bind to DNA and assist in the regulation of their target genes (Latchman, 1997), is essential to ensure the proper regulation of these events.

Transcription factors have been shown to be essential in controlling cell division (Follette and O'Farrell, 1997), regulating cell size and shape (Lecuit and Le Goff, 2007) and specifying cell identity (Mann and Morata, 2000), to list a few of their many functions. These proteins are often involved in regulating multiple processes. For example, the transcription factor Scalloped (SD) in *Drosophila melanogaster*, is required for cell proliferation (Delanoue et al., 2004; Liu et al., 2000) and differentiation (Halder et al., 1998; Kim et al., 1997; Kim et al., 1996; Simmonds et al., 1998). Furthermore, a single transcription factor can be expressed at different times and locations, and in each instance it can activate a unique set of genes. The *sd* gene is expressed at both the embryonic and larval stages of development, and the SD protein is present in a variety of different tissues from the central and peripheral nervous system (CNS, PNS respectively) to the eye-antennal, leg, and wing imaginal discs (Campbell et al., 1992). Understanding the pleiotropic nature of transcription factors is essential for our comprehension of how the embryo develops.

The work presented in this thesis helps increase our understanding of how transcription factors direct developmental events. Specifically, it will focus on the role SD plays during development as well as how SD is able to activate different sets of genes in a tissue specific manner.

### TEA/ATTS PROTEINS

The SD protein contains an evolutionary conserved TEA/ATTS domain (Campbell et al., 1992). The TEA/ATTS motif was first identified by a BLAST search using the human homolog, transcription enhancer factor -1 (TEF-1) (Burglin, 1991). To date, several different TEA/ATTS motif proteins have been identified: Tec1p in *Saccharomyces cerevisiae* (Laloux et al., 1990), ABAA in *Aspergillus nidulans* (Mirabito et al., 1989), and EGL-44 *Caenorhabditis elegans* (Mitani et al., 1993), to name a few. Electrophoretic mobility shift assays (EMSA) reveal that this region is a DNA binding domain (Xiao et al., 1991) that recognizes several motifs such as the GT-IIC (5' TGGAATGT 3') and Sph (5' ATGCATGC 3') sequences (Xiao et al., 1991) and the M-CAT elements (5'- $^{G}/_{A}$CATNC$^{C}/_{T}$ $^{T}/_{A}$-3') (Hwang et al., 1993; Stewart et al., 1994). Because the sequences of these elements and others share weak similarity, it has been suggested that TEA/ATTS proteins bind to a single highly degenerative DNA motif (Burglin, 1991; Farrance et al., 1992).

Although TEA/ATTS proteins function as transcription factors, they are generally unable to activate transcription on their own. Consequently, it is essential for TEA/ATTS domain containing proteins to interact with protein co-factors in order to promote proper activation of their target genes (Hwang et al., 1993; Vaudin et al., 1999). Secondary sequence analysis predicted that the TEA/ATTS domain contains three helices, H1, H2 and H3 (Burglin, 1991). Later, nuclear magnetic resonance (NMR) studies showed that all three of the predicted helices form an α-helix. The H1 and H2 helices run anti-parallel to each other and are separated by a 16 to 18 amino acid loop, L1. The amino terminal portion of L1 and H2 interact with each other to form a hydrophobic patch that is speculated to function as a protein docking site. The third helix, H3, lies below and roughly perpendicular to H1 and H2, and is the DNA recognition helix (Anbanandam et al., 2006). About 100 to 130 amino acids downstream of the TEA/ATTS DNA binding domain is another small block of weak sequence similarity (Burglin, 1991). Many of the protein co-factors identified, to date, interact with TEA/ATTS proteins via this second weakly conserved C- terminal region (Chen et al., 2004a; Chen et al., 2004b; Chou et al., 2006; Halder et al., 1998; Maeda et al., 2002a; Simmonds et al., 1998; Vassilev et al., 2001; Vaudin et al., 1999).

In each species studied, the TEA/ATTS domain family of transcription factors is responsible for directing the expression of several different sets of genes. In yeast, Tec1p, is responsible for activating genes involved in haploid invasive and diploid pseudohyphal growth (Gavrias et al., 1996; Roberts and Fink, 1994); EGL-44 directs FLP and HSN neuron differentiation (Wu et al., 2001). In flies SD is required for the proper formation of the adult wings, legs, eyes and the nervous system (Anand et al., 1990; Campbell et al., 1992; Garg et al., 2007; Halder et al., 1998; Inamdar et al., 1993; Simmonds et al., 1998). In mammals TEF-1 aids in skeletal, cardiac and muscle development (Chen et al., 1994; Farrance and Ordahl, 1996; Hsu et al., 1996). These observations raise the question, how does a single transcription factor regulate the expression of different genes? Work done with the TEA/ATTS proteins has provided some insight towards this question.

The yeast TEA/ATTS domain protein, Tec1p, was originally identified as a regulator of *Ty1* transposon insertion (Laloux et al., 1990). Tec1p recognizes and binds to the conserved sequences 5' CATTCC 3' and 5' CATTCT '3. These sequences are collectively referred to as the TEA/ATTS consensus sequence (TCS) (Baur et al., 1997; Hwang et al., 1993; Madhani and Fink, 1997). TCS sites have been found to be located in isolation (Chou et al., 2006), tandem to each other (Kohler et al., 2002) or adjacent to pheromone response elements (PRE) (Chou et al., 2006; Fields and Herskowitz, 1985; Kronstad et al., 1987). Enhancers containing both a TCS and a PRE site are referred to as a filamentous and invasion response element (FRE) (Madhani and Fink, 1997). Typically, TCS and FRE sites are located upstream of genes required for haploid invasive growth and diploid pseudohyphal development, respectively (Chou et al., 2006; Kohler et al., 2002). Thus, understanding how TEC1p is directed to these two enhancer types would provide some understanding on how it is able to activate two different sets of genes.

To date, only a single co-factor of Tec1p has been identified in yeast, Ste12p (Baur et al., 1997). The Ste12p protein is another transcription factor that recognizes and binds to the PRE motif (Fields and Herskowitz, 1985; Kronstad et al., 1987). EMSA experiments show that Ste12p and Tec1p can cooperatively bind to the FRE element (Madhani and Fink, 1997) and that activation of genes under the control of this element only requires the N-terminal portion of the Tec1p (Kohler et al., 2002). It has been postulated that the TEA/ATTS DNA binding domain of Tec1p interacts with the N-terminal region of Ste12p (Kohler et al., 2002). Interestingly, Ste12p is also required for the activation of several genes that contain only a single TCS but no PRE binding sites. Originally, it was postulated that this is an indirect requirement because Ste12p is necessary for the induction of Tec1p (Kohler et al., 2002). However, chromatin immunoprecipitation (ChIP) experiments show that Ste12p can associate with enhancers containing only a single TCS site. Unlike gene activation through the FRE, induction of transcription from single TCS sites requires the entire Tec1p (Chou et al., 2006). On enhancers lacking a PRE, the C-terminal region of Tec1p associates with Ste12p, allowing the N-terminal portion of Ste12p to interact with another co-factor, Dig2p. It has been postulated that the competition between Tec1p and Dig2p for the N-terminal tail of Ste12p dictates the type of Tec1p/Ste12p complex that will form (Chou et al., 2006). Tec1p can also function in a Ste12p independent manner (Kohler et al., 2002). Enhancers containing a tandem TCS binding site can activate transcription in a *Ste12* mutant background. Activation through this enhancer requires the entire Tec1p protein, indicating that Tec1p is binding to another, yet to be identified co-factor (Kohler et al., 2002).

Thus, the work done on Tec1p shows it can activate transcription in three different situations. The decision of which enhancer the TEA/ATTS protein binds to depends on whether or not Ste12p is present and the ratio of Tec1p to Dig2p. In the absence of Ste12p, Tec1p activates transcription on enhancers containing a tandem TCS (Kohler et al., 2002). In the presence of Ste12p, the choice of enhancer depends on the ratio of Tec1p to Dig2p. If this ratio allows the Tec1p to out compete Dig2p for the N-terminal region of Ste12p, a Tec1p/Ste12p complex will form on the FRE; otherwise a Tec1p/Ste12p/Dig2p complex will form on the PRE-less enhancer (Chou et al., 2006).

Although only a single co-factor has been identified in yeast, several different proteins have shown to interact with the human ATTS/TEA domain transcription factor, TEF-1. These co-activators can be divided into four classes (Mahoney et al., 2005). The p160 family of transcription factors (Belandia and Parker, 2000), yes associated protein 65 (YAP65) (Vassilev et al., 2001), and the YAP65 related transcriptional co-activator with PDZ-binding motif (TAZ) (Mahoney et al., 2005) proteins all belong to the first class of co-factors. These proteins are unable to bind to DNA and activate transcription from MCAT (5' CATTCCT 3') enhancers. The second class consists of the Vestigial-like protein family; Vgl-1; Vgl-2; Vgl-3 and Vgl-4 (Chen et al., 2004b; Maeda et al., 2002a; Vaudin et al., 1999). The Vgl set of co-factors are also unable to bind DNA or activate transcription, and they are expressed in a tissue-specific manner. They are required for the proper formation of the placenta, skeletal muscles and the heart. DNA co-binders form the third class of TEA/ATTS co-factors. These proteins form a tertiary complex with TEF-1 and may be involved in chromatin remodeling. Only a single protein from

this class of co-factors has been identified, the nuclear protein poly(ADP- ribose) polymerase (PARP) (Butler and Ordahl, 1999). The final class includes other transcription factors. Proteins belonging to class IV include the basic-helix-loop leucine zipper protein Max(Gupta et al., 1997), the myocyte enhancer factor -2 (MEF-2) (Maeda et al., 2002b) and the serum response factor (SRF) (Gupta et al., 2001). Because TEF-1 has been shown to interact with a large repertoire of co-factors, it is possible the interaction with different binding partners helps direct Tef-1 to specific enhancers. Indeed, work done with each of these co-factors helps support this idea.

The SRF protein contains a highly conserved DNA binding domain that belongs to the MADS box family of transcription factors that recognize and bind to a 5' CC(A/T)$_6$GG 3' serum response element (SRE) (Shore and Sharrocks, 1995). SREs are generally found in close proximity to other consensus binding sites (Gilman et al., 1986; Greenberg et al., 1987), giving rise to the paradigm that these other transcription factors help determine the transcriptional status of SRF regulated genes. The fact that MCAT binding sites are often found in conjunction with SRE sites in genes specifically expressed in cardiac myocytes (MacLellan et al., 1994; Zhu et al., 1991) led to the idea that SRF might be able to interact with TEF-1. *In vitro* and *in vivo* studies were able to confirm this prediction. Transfection of COS1 cells with reporter constructs carrying both the MCAT and SRE binding site was able to induce the expression of the luciferase gene. However, mutating either of these binding sites resulted in a return to basal levels of expression (Gupta et al., 2001). The requirement of SRF for proper induction of transcription from this enhancer element may be to help stabilize the association of TEF-1 with DNA. Work done in yeast supports this idea. Hiten *et al.* showed in an EMSA experiment that only when Tec1p and Ste12p are present together are they able to supershift a *Ty1* FRE motif (Madhani and Fink, 1997). Furthermore, they showed DNase I foot printing experiments that revealed a pair of adjacent hypersensitive sites between the TCS and the PRE elements; suggesting that Tec1p and Ste12p may be cooperatively binding to each other by causing distortions in the DNA.

Glutathione *S*-transferase (GST) pull-down experiments have shown that the nuclear phosphoprotein, Max, also interacts with Tef-1 (Gupta et al., 1997). Max, a basic helix-loop-helix leucine zipper protein that recognizes the E-box motif, has traditionally been shown to be a binding partner of Myc (Blackwood and Eisenman, 1991). Max has also been shown to form homodimers with itself and heterodimers with other basic helix-loop-helix leucine zipper proteins (Berberich and Cole, 1992). Work done with the α-MHC gene, a gene that contains both an E-box and an MCAT binding site in its enhancer, shows that expression of this gene is repressed in cells expressing only Max or TEF-1. However, when these two proteins are present together, the repression is alleviated and the downstream gene is turned on. Furthermore, it was shown that this activation is dependent on the amount of Myc and TEF-1. As the ratio of Myc to TEF-1 deviated from 1:1, the activation properties of the complex decreased (Gupta et al., 1997). Thus, Gupta *et al.* have been able to demonstrate that TEF-1 can function as a repressor. Furthermore, they also show that TEA/ATTS cofactors can alter whether TEF-1 functions as an activator or a repressor.

Early work done on TEF-1 showed that the regions outside of the TEA/ATTS domain are also important for proper sequence recognition. Deleting either the C-terminal 34 amino acids or a serine-threonine-tyrosine (STY) rich region (a.a. 306 – 328) eliminates the ability of TEF-1 to recognize and bind to the GTIIC motif *in vitro*. Presumably, these ~~regions are required to antagonize~~ an inhibitory domain of TEF-1. Furthermore, despite the fact the TEA/ATTS domains of TEF-1 and SD only differ by a single amino acid, SD is unable to interact with either the SphI or the GTIIC element. Altering the SD TEA/ATTS sequence to the TEF-1 sequence only minimally restores the SD protein's ability to recognize the GTIIC motif and has no effect on its ability to bind to the SphI element (Hwang et al., 1993). Despite the fact the complete SD protein does not bind to the GTIIC motif, the TEA/ATTS domain of SD on its own is able to associate with the GTIIC sequence (Halder and Carroll, 2001). Although SD was originally shown not to bind to a GTIIC element (Hwang et al., 1993), it was later shown to recognize the motif, *in vitro* (Halder and Carroll, 2001). If these results hold true *in vivo*, one can postulate that the manner by which TEA/ATTS co-factors interact with regions outside the DNA binding domain influences the enhancer elements that TEA/ATTS domain proteins recognize.

The SD protein is able to interact with the co-factor vestigial (VG) (Halder et al., 1998; Simmonds et al., 1998). Unlike the two previously described TEA/ATTS co-factors, VG is unable to bind DNA. Halder *et al.* have shown, mainly through *in vitro* experiments, that the SD protein recognizes two different motifs, A sites and B sites (See Table 1.2). The A sites occur singly or as doublets, while the B sites are found exclusively in the doublet configuration. Furthermore, they show that SD not bound by a co-factor preferentially bind to A sites. This preferential affinity is lost when SD interacts with VG. Only the region of VG that directly facilitates binding to SD is required to alter SD DNA binding specificity. In addition to altering the ability of SD to bind to A sites, the binding of VG to SD promotes the formation of a higher order tetrameric structure consisting of two SD and two VG proteins. Although the N and C terminal regions of VG cannot co-immunoprecipitate SD, they are required for the formation of the tetrameric structure (Halder and Carroll, 2001). This idea of VG altering the ability to recognize A sites is also seen with work done with the Vgl-2 protein. Chen *et al.* noticed, despite the fact TEF-1 expression levels remain constant, that the amount of TEF-1 that associated with the MCAT enhancer decreased as skeletal differentiation progressed. Furthermore, they were able to show through EMSA that the ability of TEF-1 to bind to the MCAT enhancer decreased with increasing levels Vgl-2 (Chen et al., 2004a). Thus, the loss of TEF-1 binding to the MCAT enhancer may be due to the fact that Vgl-2 expression levels are up regulated during skeletal differentiation (Gunther et al., 2004).

In addition to TEF-1, three other mammalian TEA/ATTS domain proteins have been isolated (Andrianopoulos and Timberlake, 1991). TEF-3 (also referred to as RTEF-1 in humans or TEAD-4 in mice) was originally identified as FR-19, a fibroblast inducible gene that promotes the differentiation of myoblasts to myotubes. It is also heavily expressed in lungs and liver (Hsu et al., 1996). TEF-4 (ETF-1 or TEAD2) is the first TEA/ATTS protein expressed and is first detected at the two cell stage (Kaneko and DePamphilis, 1998). In addition to TEAD2 being required for early mouse

embryogenesis, it is also important for proper skeletal formation (Kaneko et al., 1997; Yockey et al., 1996). The last mammalian TEA/ATTS domain protein, TEF-5 (DTEF-1 or TEAD3), is expressed in the placenta and is important for fetal development (Jacquemin et al., 1997; Jacquemin et al., 1998; Jiang et al., 1999). Although all four mammalian TEF proteins contain identical TEA/ATTS domains (Jacquemin et al., 1996) and are able to bind to the same enhancers with similar affinity (Kaneko and DePamphilis, 1998), they do not appear to be functionally redundant. This is seen by the fact that deletion of TEAD1 causes lethality at stage 11 of development (Chen et al., 1994), even though the other TEAD proteins are expressed in every TEAD1 expressing cell by stage 8 (Kaneko et al., 1997). These observations further complicate our original question as to how transcription factors with identical DNA binding domains regulate the expression of different genes. Recall, the Tec1p protein is able to recognize different enhancer elements depending on the type and amount of co-factors present. Thus, it is possible that the TEF family of proteins is also able to regulate the expression of a unique set of genes by differentially responding to co-factors (Kaneko and DePamphilis, 1998). This idea is supported by the work done with MEF-2, Yap65, TAZ and PARP.

The interaction domains of SRF and TEF-1 have been identified. This interaction occurs via the MADS box DNA binding domain of the SRF. MEF-2 is another transcription factor that contains a MADS box domain (Black et al., 1996). It should be noted that recently, the *Drosophila* homolog of TEF-1, *sd*, has also been shown to form a transcription factor complex with dMEF-2 (Personal communication with Hua Deng). The MADS box of MEF-2 and SRF are 50% identical (Alvarez-Buylla et al., 2000). This observation, plus the fact that MEF-2 is required for muscle development (Black and Olson, 1998), suggested that TEF-1 and MEF-2 may interact with each other. Indeed, GST pull-down experiments confirmed this interaction. However, unlike SRF, the entire MEF-2 protein and TEF-1 protein are required for optimal induction of the reporter construct. This observation suggests that regions outside the MADS box are required for MEF-2 to interact with TEF-1. Reporter studies using the MEF2 enhancers MLC2v and βMHC also revealed that each of the TEF-1 orthologs differentially regulate these two enhancers. TEF-1 and TEF-3 are able to squelch expression from the MLC2v element, while TEF-5 had no effect. In contrast, TEF-3 and TEF-5 were able to enhance expression from the βMHC reporter. Because both of these promoters contain a SRE and MCAT binding site, these results not only suggest that other transcription factors or co-factors are required for proper activation of downstream target genes, but that each TEF isoform may have a different propensity to bind to each of these protein partners (Maeda et al., 2002b). Support for this hypothesis is seen with the work done with the YAP65 and TAZ binding partner. The two PDZ binding motif-containing co-activators YAP65 and TAZ have both been shown to interact with TEF-1 (Mahoney et al., 2005; Vassilev et al., 2001). In addition to having a PDZ domain, both co-factors have at least one WW domain and a 14-3-3 binding site (Kanai et al., 2000). Unlike interaction with other proteins, the WW domain of YAP65 and TAZ is not required for them to interact with TEF. This observation suggests that when these two PDZ proteins are bound to members of the TEF family they could potentially recruit other co-factors (Mahoney et al., 2005; Vassilev et al., 2001). Although YAP65 and TAZ can bind to all four mammalian TEF proteins, they were found to preferentially bind to TEF-4 and TEF-1, respectively

(Mahoney et al., 2005; Vassilev et al., 2001). It is possible that this preferential binding might be even further enhanced *in vivo*.

Another key feature about YAP65 and TAZ proteins is that their cellular localization is controlled by phosphorylation. Phosphorylation of the YAP65 and TAZ proteins causes them to be retained in the cytoplasm, while dephosphorylation results in nuclear localization (Vassilev et al., 2001). Consequently, the availability of these two co-factors to interact with TEF transcription factors can be regulated. Thus, it has been hypothesized that the combination of preferential binding of different class I and class II co-factors to different TEF family members, the possibility of these binding partners to potentially recruit other proteins, and the ability to regulate the cellular localization of these co-factors may be methods by which target specificity is achieved (Mahoney et al., 2005).

In addition to preferentially binding to different co-factors, these binding partners have been shown to exhibit different transcription properties depending on the isoform with which they associate. TEF-1 has been shown to form two different transcription complexes on the MCAT1 and MCAT2 enhancers (Larkin et al., 1996). One of the proteins found to be part of the complex on the MCAT1 element is the nuclear PARP protein (Butler and Ordahl, 1999). PARP is a DNA binding protein that was originally shown to be involved in DNA break repair (Alkhatib et al., 1987; Bradbury, 1992; Wang et al., 1995). This nuclear protein is only able to bind to the MCAT1 motif and not the MCAT2 motif. Not only is the PARP protein able to interact with DNA, but it is also able to bind to and ADP-ribosylate the TEF family of proteins. Furthermore, inhibition of PARP activity reduces the activity of the MCAT1 enhancer (Butler and Ordahl, 1999). These observations have led to the idea that interaction with PARP proteins may be a different way that TEF members are able to activate various sets of genes. PARP can function as either an activator or inhibitor of transcription. Thus, depending on the TEF member that PARP is bound, this might influence whether a gene is expressed (Butler and Ordahl, 1999). It should be noted that PARP has been shown to bind to a plethora of transcription factors (Kraus and Lis, 2003), and thus PARP may function in a superficial manner in the process of gaining tissue specificity.

The work done with each of the TEA/ATTS members has already provided some insight into how a single transcription factor is able to regulate the activity of various downstream target genes. Target specificity can be enhanced by association with other transcription factors. This can be done in two ways. The first is by directing the complex to different enhancers. For example, the TEF-1\SRF complex will only be able to associate with enhancers containing both the SRE and MCAT motifs (Gupta et al., 2001), while the TEF-1\Max dimer will only bind to DNA with an E-box and MCAT motif (Gupta et al., 1997). The second method, as seen in the Tec1p/Ste12p story, is through a synergistic interaction, whereby the co-factors enhance the ability of the TEA/ATTS proteins to associate with low affinity TCS sequences (Madhani and Fink, 1997). In addition to enhancing the affinity for DNA, co-factors can also alter the enhancers that TEA/ATTS proteins recognize. This was demonstrated with the work done on the class II protein, VG, where it was shown that the VG protein alters the ability of TEA/ATTS

proteins from binding A sites to B sites (Halder and Carroll, 2001). Finally, co-factors can change the transcriptional properties of the TEA/ATTS proteins.

This method is supported by the work done with the PARP protein (Butler and Ordahl, 1999). It should be noted that these methods of gaining enhancer specificity are not restricted to TEA/ATTS proteins, but can also be seen with other transcription factors. For example the Homeodomain containing HOX genes utilize many of the above described methods to gain tissue specificity. The interaction of the HOX protein, Labial (LAB), with the transcription factor extradenticle (EXD) has been shown to alter the binding sites that LAB recognizes (Chan et al., 1996). Furthermore, the interaction of EXD to other HOX proteins can also lead to enhanced specificity for binding DNA (Chan and Mann, 1996). Finally, the recruitment of Sloppy-paired and Engrailed proteins to the enhancer can alter the transcription state of the HOX-EXD complex (Gebelein et al., 2004).

PROTEIN: PROTEIN INTERACTION DOMAIN

Identifying the domains that the TEA/ATTS proteins and their co-factors use to interact with each other has not only facilitated the discovery of novel co-factors, but also increased our understanding of how enhancer specificity is achieved. The identity of one of these domains comes from work done on the MADS family of transcription factors. Both the SRF and Max proteins use the MADS DNA binding domain to interact with TEF-1 (Gupta et al., 2001; Gupta et al., 1997). Furthermore, introducing point mutations in helices H2 and H3, but not in H1, of the TEA/ATTS domain perturb this association (Gupta et al., 2001). Thus, in accordance with the NMR studies, the MADS domain probably interacts with the hydrophobic pocket created by the L1 loop and the H2 helix (Anbanandam et al., 2006). In addition to co-factors interacting with the DNA binding domain, they are also able to interact with the C-terminal end of the of TEA/ATTS proteins (Chen et al., 2004b; Maeda et al., 2002a; Vassilev et al., 2001; Vaudin et al., 1999). The C-terminal end of the protein is composed of a region of weak sequence similarity, between the TEAD homologs, (Burglin, 1991), a presumptive zinc finger, as well as a STY and a proline rich domain. Deletion studies revealed that the VG and VGL family of co-factors require both the region of weak sequence similarity and the STY rich domain to properly interact with TEA/ATTS domain proteins (Chow et al., 2004; Maeda et al., 2002a; Vaudin et al., 1999). These two regions collectively make up the vestigial interaction domain (VID). In contrast, the YAP65 co-factor requires the entire C-terminal end of TEF-1. Deletion of any of the domains C-terminal to the TEA/ATTS domain, except the proline rich region, causes a complete loss of the ability of TEF-1 to co-immunoprecipitate YAP65. Because removal of the proline rich domain causes partial loss of binding, this region is also considered important for TEF-1 to interact with YAP65. The entire C-terminal region of the protein that is required to facilitate the interaction between YAP65 and TEF-1 is referred to as the transactivating domain (Vassilev et al., 2001). Located in the C-terminal half of TEA/ATTS proteins, a region of 55 amino acids, the scalloped interaction domain (SID), has been shown to be essential for VG to interact with SD. Alignment using the SID from VG and Vgl-1 reveals a stretch of 9 amino acids that have been perfectly conserved (Simmonds et al.,

1998). This sequence, VDEHFSRAL, was critical in the identification of Vgl-4, a protein that contains two domains with this sequence (Chen et al., 2004b). A stretch of 107 amino acids located in the N-terminal half of YA65 is essential for it to interact with SD. Although this domain interacts with a region of SD that contains the VID, the VDEHFSRAL motif is not evident within the YAP65 sequence. The absence of the VDEHFSRAL motif may explain reason why the YAP65 protein requires regions outside the VID to properly interact with TEA/ATTS proteins.

VESTIGIAL

Both SD and VG were originally shown to be critical for the proper development of the wings and halteres (Williams et al., 1991). Expression pattern studies showed that VG and SD are expressed in a similar pattern in the wing imaginal disc (Campbell et al., 1992; Williams et al., 1994), leading to the idea that these two proteins likely are able to interact with each other (Halder et al., 1998; Simmonds et al., 1998). To date, VG is the only published co-factor for SD. Because co-transfection of SD and VG in S2 cells can activate three different dSRF enhancers, it was proposed that the role of VG is to activate transcription (Halder et al., 1998). In support of this idea, fusion of either the N-terminal or the C-terminal domains of VG to a LexA DNA binding domain is also able to activate transcription of the *LEU2* gene in yeast (Vaudin et al., 1999). However, recent evidence casts doubt on whether VG can activate transcription on its own. Even though Vgl-2 can substitute for VG, in the context of wing development (Vaudin et al., 1999), neither Vgl-2 nor any of the other vestigial like proteins are able to activate transcription in cell culture experiments (Gunther et al., 2004; Maeda et al., 2002a). Furthermore, the SD/VG tetramer was not able to induce expression from a synthetic enhancer containing two tandem B sites in the wing imaginal disc (Guss et al., 2001). These results complicate our understanding of the role that VG is playing.

Even though it is uncertain if VG can activate transcription, *in vivo* data have revealed that both the N and C-terminal domains are required for proper wing development. Deletion of either of these regions prevents the protein from properly forming the wing and inducing the expression of downstream wing target genes (Garg et al., 2007; MacKay et al., 2003). Furthermore, deletion of only one of these domains is able to rescue wing development very poorly in a *vg* mutant background (MacKay et al., 2003). In the wing imaginal disc, the SD/VG tetramer is able to induce the expression of SD (Halder et al., 1998; Simmonds et al., 1998) and wingless (WG) (Liu et al., 2000) in a cell autonomous and cell non-autonomous manner, respectively. Ectopic expression of full length VG is able to induce the expression of both of these genes, while deletion of either the N-terminal or the C-terminal domain reduced the ability of the protein to activate the *sd*LacZ gene and completely eliminated the induction of WG.

Interestingly, the ability of VG to induce expression from a *sd*LacZ gene is hampered more when the N-terminal domain is removed, than when the C-terminal domain is deleted (MacKay et al., 2003). In a similar fashion, the ability of the SD/VG tetramer to form on DNA is more sensitive to deletions in the N-terminal domain than the C-terminal domain (Halder and Carroll, 2001). Thus, it is possible that role of the N and C-terminal

domains is not to activate transcription, but to promote the formation of the tetramer and direct the TEA/ATTS DNA binding site to the proper enhancers. In addition to directing the complex to the appropriate enhancer, the two terminal domains may be required to help facilitate the acquisition of other protein co-factors. As previously mentioned, the SD/VG complex was unable to activate transcription from synthetic enhancers containing two tandem B sites in the wing imaginal disc. In order to get induction from the enhancer, the B sites need to be placed adjacent to signaling pathway transcriptional effector binding sites such as either the Su(H) site (Notch pathway) or a Mad/Madea site (decapentaplegic pathway). Furthermore, although the SD/VG complex was able to induce expression from both these enhancers, the pattern of expression from each enhancer differed. The substitution of two Su(H) and the Mad/Medea sites for a B site, induced expression of the downstream genes along the dorsal/ventral boundary and in each of the quadrants of the wing imaginal disc, respectively. The differential expression seen by the two enhancers is due to the differential expression of Su(H) and Mad/Medea rather than the functional state of the SD/VG complex. It should be noted that the presence of only the Su(H) or the Mad/Medea binding sites is also unable to activate transcription (Guss et al., 2001).

Why activation of the enhancers requires two different transcription factors is unclear. One hypothesis is that the SD/VG complex may interact with the adjacent transcription factor complex, to help increase its stability to DNA. However this idea is unlikely, because *in vitro* experiments show the SD/VG tetramer is able to bind to B sites effectively (Halder and Carroll, 2001). Furthermore, this explanation would require the SD/VG complex to directly interact with many different signaling pathways. A second hypothesis is that both transcription factors are required to recruit the necessary basal transcription machinery and chromatin remodeling factors (Guss et al., 2001). This requirement can be met in two ways. One method is that each transcription factor may recruit a specific subset of these proteins. Hence, only when these transcription factors are bound to DNA, in close proximity to each other is the complete set of co-factors present. The second is that stable recruitment of basal transcription machinery and chromatin remodeling proteins requires both transcription factors. This last method is supported by the observation that the C-terminal binding protein (CBP) is able to directly bind to signaling pathway transcription effectors and to SD (Goodman and Smolik, 2000; Guss et al., 2001). Thus, even though each transcription factor is able to bind to CBP *in vitro*, stable recruitment of this protein *in vivo* requires it to interact with both families of transcription factors. If this latter model is correct, it could potentially explain the discrepancies reported in the literature in regards to the transcriptional activation properties of VG. Many of the assays done to determine the activational properties of VG and Vgl proteins were done in various cell lines. Different cell lines would have varying levels of expression for different proteins. Consequently, if a protein such as CBP is expressed at a high enough level in a particular cell line, the requirement for both transcription factors to be present to recruit it to the enhancer may be bypassed.

SCALLOPED

The *Drosophila melanogaster* genome contains a single TEA/ATTS gene, *sd*, that maps to position 13F1- 13F4 on the X chromosome (Campbell et al., 1991). Several lethal alleles have been mapped to this region. These lethal alleles can be divided into two categories. The first class contains nonsense point mutations and deletions. Alleles that fall under this category are lethal as embryos or 1$^{st}$ instar larvae. The second class is composed solely of missense point mutations and cause lethality at the late pupal stage of development. Class II mutations are distributed throughout the *sd* gene. They have been mapped to nucleotides encoding for an amino acid between helices H2 and H3 of the TEA/ATTS DNA binding domain, to a tyrosine just downstream of the VID, and to a histidine at the C-terminal end of the protein. The fact that different mutations cause lethality at different stages, and that class II alleles have propensities to complement hypomorphic alleles of *sd*, further re-enforces the idea that different regions of the SD protein are required for different developmental processes (Srivastava et al., 2004).

In the embryo, *sd* is primarily expressed in sense organ cells. Expression studies using an enhancer trap allele of *sd*, $sd^{ETX4}$, and a cDNA probe reveals that, at stage 9, the *sd* gene is initially expressed in the cephalic neuroblasts and by stage 11 its expression is expanded to include the PNS. At the end of stage 16, the *sd* transcript is also present in the dorsal peripheral sense organs, the lateral and ventral sense organs in the trunk region, the antennomaxillary complex and the clypeolabral sense organs. Embryos homozygous for class I alleles of *sd* have abnormalities in the number and position of sense organ cells (Campbell et al., 1992). In addition to being expressed in neural cells, SD is also present in embryonic muscle precursor cells. Ectopic expression of SD in the embryo causes an increase in the number of cells expressing a muscle specific marker *ladybird* (Bidet et al., 2003). In larvae, the *sd* transcript is expressed in the optic lobe and each of the imaginal discs except the labial disc (Campbell et al., 1992). Most of the work with SD centers on its role in wing development. In the wing imaginal disc, *sd* is predominantly expressed in the wing pouch, and the lateral regions of the dorsal hinge (Campbell et al., 1992).

Proper function of SD in the wing imaginal disc requires its binding partner VG (Halder et al., 1998; Simmonds et al., 1998). Together these two proteins form a selector complex. Selector proteins/complexes are transcription factors that are able to direct the tissue identity of a cluster of cells (Garcia-Bellido, 1975). They are able to accomplish this by activating the appropriate set of downstream target genes that are required to pattern the tissue. For example, the selector genes *homothorax* (*hth*) and *exd* together specify ventral leg appendage. The absence of either of these genes in the leg imaginal disc results in a loss of the appendage, while the ectopic expression of HTH and EXD can lead to formation of exogenous legs (Casares and Mann, 1998). In a similar fashion, not only are SD and VG required for proper wing development, but the presence of both of these proteins in the eye imaginal disc leads to the induction of wing tissue outgrowths (Kim et al., 1996). The SD/VG complex has also been shown to induce the expression of genes required to sub pattern the wing, such as *cut, spalt, knirps,* and *drosophila serum response factor* (Giot et al., 2003; Halder et al., 1998). Furthermore, the fact that SD/VG binding sites reside in the enhancers of each of these downstream genes suggests

that the SD/VG complex directly induces the expression of them (Guss et al., 2001; Halder and Carroll, 2001). In addition to inducing the expression of downstream target genes, the SD/VG complex is also required for cell survival and proliferation. This requirement is specific to cells that reside in the wing pouch (Liu et al., 2000). In the wing pouch, the wing selector complex has been shown to antagonize the effect of *decapo*, a cyclin-cdk inhibitor, and induce the expression of genes involved in cell cycle progression (Delanoue et al., 2004; Legent et al., 2006).

Although a lot of work has been done to try to understand how TEA/ATTS proteins function and how target specificity is achieved, several questions still remain unanswered. The interaction of SD with VG has previously been shown to direct the SD TEA/ATTS domain to alter the DNA motif it recognizes, yet how VG causes this change is still unclear. Is there actually a distinct sequence difference between A sites or B sites, or are B sites just tandem A sites? If B sites are just tandem A sites, then how does the binding of VG direct the complex to B sites but not A sites? Furthermore, the functional roles of the two terminal domains of VG still need to be clarified. Are these regions able to activate transcription? Or do they assist in the recruitment of other co-factors? Finally, outside of the wing disc very little is known about the functional roles of SD in other tissues. Since wings are not required for the survival of lab stocks, the presence of lethal alleles suggests a critical wing-independent role for SD. What is the role of SD in these tissues? Does SD interact with other co-factors in a tissue specific manner? Despite the fact several different co-factors have been shown to associate with TEF-1, until recently only a single binding partner has been identified for SD. If SD indeed requires other co-factors to promote the development on non-wing tissues, what is the identity of them? Through the use of a variety of molecular and biochemical techniques, this thesis will provide some insight into these questions.

Table 1.1. TEAD Proteins in each species, and their respective binding partners.

| Species | TEAD Proteins | Binding Partners |
|---|---|---|
| Yeast | Tec1p | Dig2p; Ste12p |
| Drosophila | SD | VG; Yorkie; dMEF-2 |
| Vertebrates | TEF-1, TEF-2, TEF-3, TEF-4 | p160; Max; MEF-2; PARP; SRF; TAZ; VGL-1; VGL-2; VGL-3; VGL4; YAP65 |

References for each of the binding partners are found within the text of the introduction.

Table 1.2. List of the identified A sites and B sites that the SD protein recognize

| A Sites | B Sites |
|---|---|
| CATA**ACTTATTA**AAAA | TCAA**TGTAATTCGAAAAATGT**CGTC |
| AGAG**AGGAATGC**AACA | CAGA**TAAAATTATTGAAATTA**CATT |
| CACG**TGGAATGA**GCTA | TTTC**TGGAATCCCACGAATGT**CCAT |
| CTTG**TGGAATGT**GTTT | CCTC**TTACATTTGTCGCATAG**TTCC |

The DNA sequence the SD protein interacts with are in bold.

References of the sites are found within the text of the introduction

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**CHAPTER II - Antagonizing scalloped with a novel vestigial construct reveals an important role for scalloped in Drosophila melanogaster leg, eye and optic lobe development**[1]

## INTRODUCTION

Throughout the course of development a single transcription factor may often be used to control the patterning of different tissues. For example, in *Drosophila melanogaster*, the paired domain protein Eyeless is required for the proper development of the adult compound eye (Halder et al., 1995; Sheng et al., 1997) as well as of the adult central nervous system (Callaerts et al., 2001). The Drosophila TEA/ATTS domain (TEAD) protein, Scalloped (SD), is a transcription factor that is expressed in several different tissues throughout development. The existence of lethal alleles of *sd* (Campbell et al., 1992; Campbell et al., 1991) implies that this gene is vital for proper development.

The TEAD is a highly conserved DNA-binding domain that recognizes the M-CAT motif (5'-TCATTCCT-3') (Hwang et al., 1993; Stewart et al., 1994). TEAD-containing proteins generally bind directly to tissue-specific transcriptional intermediary factors (TIFs) to properly function as a specific transcription factor (TF) complex (Chen et al., 2004a; Chen et al., 2004b; Halder et al., 1998; Jiang et al., 2000; Maeda et al., 2002; Mahoney et al., 2005; Simmonds et al., 1998; Vaudin et al., 1999). Since TEAD-containing proteins, such as SD, often lack an activation domain (Hwang et al., 1993; Vaudin et al., 1999), the associated TIFs may provide this function (Chen et al., 2004a; Chen et al., 2004b; Halder et al., 1998; Jiang et al., 2000; Maeda et al., 2002; Mahoney et al., 2005; Simmonds et al., 1998; Vaudin et al., 1999). Alternatively, the activation domains of TEAD-containing proteins may associate with TIFs that can act as coactivators or corepressors. To date, two TIF-interacting domains have been identified for TEAD proteins: the Vestigial interacting domain (VID) (Halder et al., 1998; Simmonds et al., 1998) and the C-terminal YAP/TAZ-transactivating domain (TD) (Vassilev et al., 2001). GST pull-down experiments with the human homolog transcription enhancer factor-1 (TEF-1) loosely position the VID and the TD between amino acids 221 and 329 (Chow et al., 2004) and 224 and 426 (Vassilev et al., 2001), respectively. The VID interacts with the Vestigial protein (VG) (Halder et al., 1998; Simmonds et al., 1998) and with Vestigial-like proteins (VGL) (Chen et al., 2004a; Chen et al., 2004b; Halder and Carroll, 2001; Mielcarek et al., 2002; Vaudin et al., 1999) in flies and mammals, respectively. The TD has been shown to be functionally important to interact with the yes-associating protein 65 (YAP65)/Yorkie (Vassilev et al., 2001; Wu et al., 2008; Zhang et al., 2008), and the Yap65 homolog TAZ (Mahoney et al., 2005).

The gene *scalloped* (*sd*) is the only TEAD-encoding gene in the Drosophila genome. Enhancer trap studies reveal that *sd* is first expressed at stage 14 in the peripheral nervous system (PNS), the antennomaxillary complex, and the supraesophageal ganglion. By

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Co-author Ajay Srivastava created the VGΔACT construct
Sandra O'Keefe and Monica Davis did the micro injection of the construct
Leola Chow created and microinjected the SDΔ200 construct

stage 16, *sd* expression is expanded to the anterior sense organs and the sense organs of the gnathal regions (Campbell et al., 1992). SD is also thought to be expressed in embryonic cardiac cells (Bidet et al., 2003). In third instar larvae, *sd* is present in the optic lobes and in a few discrete cells of the cerebral hemisphere and the ventral nerve cord. In the wing imaginal disc, *sd* is expressed in the wing blade, scutellum, and the mesopleura, while in the eye imaginal disc, staining is restricted to cells behind the morphogenetic furrow (Campbell et al., 1992).

Most of our understanding of SD function is derived from work done with the wing imaginal disc, where SD interacts with the TIF, VG (Halder et al., 1998; Simmonds et al., 1998). VG does not contain a DNA-binding motif, but contains a SD-interacting domain (SID) and two regions important for activation of downstream genes, located at the N- and C-terminal ends of the protein (MacKay et al., 2003; Vaudin et al., 1999). These three regions are required for the proper development of the wing blade (MacKay et al., 2003). Binding of SD to VG is necessary for the formation of a functional transcription complex that is able to specifically activate the expression of downstream wing genes (Halder et al., 1998; Simmonds et al., 1998). Ectopic expression of VG in cells expressing SD but not VG causes activation of downstream wing genes and directs the developmental fate of that tissue into a wing (Halder et al., 1998; Kim et al., 1996). SD is also required to localize VG to the nucleus (Halder et al., 1998; Simmonds et al., 1998; Srivastava et al., 2002). *In vitro* experiments have shown that the binding of VG to SD can alter the DNA motif that SD recognizes. These experiments have also shown that the interaction of a truncated form of VG, containing only the SID, with SD is able to disrupt the complex from binding to wing-specific DNA-binding motifs. Proper recognition of the wing-specific DNA-binding motifs by SD requires the SID and at least one of the VG domains involved in activation (Halder and Carroll, 2001). This is consistent with the findings that the protein encoded by a fusion of the *sd* TEAD domain with a full-length *vg* gene can rescue *sd* and *vg* wing mutations (Srivastava et al., 2002). In the absence of VG, SD is still able to bind to DNA, but recognizes a different motif (Halder and Carroll, 2001).

The function of SD in non-wing tissues is poorly understood. Several lethal alleles of *sd* have been isolated (Campbell et al., 1991). The lethal alleles of *sd* fall into two classes: embryonic and pupal lethal (Campbell et al., 1991; Srivastava et al., 2004). Since wings are not essential to the survival of laboratory stocks, SD likely plays a vital role in non-wing tissues. To gain a better understanding of the role that SD plays in development, we generated mitotic clones using an embryonic lethal allele of SD. Loss of SD caused blistering in the wings (Liu et al., 2000), alterations in eye bristle shape and patterning, and truncation of the legs. We further assessed the role of SD by antagonizing its function with a novel allele of VG. By expressing a truncated form of VG, VGΔACT (Figure 2.1), that is able to bind to SD but not activate transcription, we were able to recapitulate all the phenotypes observed with the flip-in clones as well as cause defects in the optic lobe and promote fusions between ommatidia. Despite the VGΔACT protein's ability to antagonize SD in larval tissues, it did not affect the development of the embryonic PNS, central nervous system (CNS), or cardiac cells. To test whether the phenotypes seen in the affected tissues are due to the inability of SD to form a functional

TF, we also expressed a truncated form of SD, SDΔ200 (Figure 2.1), that is unable to bind DNA but retains the TIF-interacting domains (Chow et al., 2004). The SDΔ200 protein should be able to bind to any TIFs that normally interact with the C-terminal 244 amino acids of the SD protein. Expression of the SDΔ200 protein induces phenotypes similar to those caused by the flip-in clones and the VGΔACT protein, indicating that SD likely binds to currently unknown TIFs in the eye, leg, and optic lobe. Through these studies we show that the level of SD is important in these tissues and that the SD/TF complex is involved in promoting cell survival in the leg imaginal disc.

## MATERIALS AND METHODS

*Drosophila stocks:*

All crosses were done at 25°. The UASsd, UAS SDΔ200, UASsdΔ88-123, and UASsdΔ88-159 constructs are described in Chow et al. (2004). The *ptc* Gal4 and *vg* Gal4 strains were a gift from S. Carroll, the $sd^{47M}$ FRT 18A was a gift from K. Irvine, and *w+, ry+* 2[p-Myc] FRT18A; *Sb* FLP/*Tb* was a gift from S. Hughes. The 24BGal4, 109(2)80Gal4, 167YGal4, 179YGal4, C147Gal4, c698aGal4, ActinGal4, *ato* Gal4, *Cha* Gal4, CQ2Gal4, *Dll* Gal4, *eye* Gal4, GMRGal4, Pan-R7Gal4, *Pdf* Gal4, RN2Gal4, $sd^{72b}$, and *sev*EPGal4 stocks were obtained from the Bloomington Stock Center. The $sd^{72b}$ allele is a deficiency stock containing a deletion that spans the entire *sd* gene.

*Flip-in clones:*

The $sd^{47M}$ allele is a 157-bp deletion (Srivastava et al., 2004) and homozygous individuals exhibit an embryonic lethal phenotype (Campbell et al., 1991). The $sd^{47M}$ allele was recombined onto a w, P(ry, neoFRT) 18A chromosome (Liu et al., 2000). Mitotic clones of this allele were generated by flipase-mediated mitotic recombination (Golic and Lindquist, 1989; Xu and Rubin, 1993) by repeated daily heat shocking at 39°C for 45-min intervals.

*Construction of VGΔACT:*

Using a polymerase chain reaction (PCR) protocol, the nucleotides that encode amino acids 171–335 of VG were amplified with the following primers: 5'-TCG AGG CCT CAC ACA CAC ACG CAT ACG-3' and 5'-GGG CTC GAG TTA GTG CAC GTA ATT GCT GTT-3'. The PCR products were digested with *XhoI* and *StuI* and cloned into a p131 vector (Abu-Shaar et al., 1999).

*PCR conditions:*

A Taq:Pfu (20:1) mix was used in standard PCR conditions. The template was allowed to initially denature at 94° for 5 min. DNA was amplified with 30 cycles at 94° for 30 sec (denaturing) followed by 55° for 30 sec (annealing) and 68° for 90 sec (extension).

*Micro-injections:*

Micro-injections were performed as described in Rubin and Spalding (1982) using the VGΔACT construct in pUAST and a helper Δ2-3 plasmid provided by S. Campbell. Four independent transgenic lines were isolated and tested. All the results shown make use of the VGΔACT-22 line.

*Scanning electron microscopy:*

Adult flies were fixed in a 2% glutaraldehyde, 1x PBS solution for 1 hr at room temperature. Samples were washed twice in 1x PBS before being dehydrated in increasing concentrations of ethanol. Ethanol was removed by bathing the samples in increasing concentrations of hexamethyldisilazane:ethanol solutions. Samples were dried and gold coated before visualization by scanning electron microscopy (SEM).

*Silver staining:*

Adult flies were fixed overnight in a 10% formalin solution, dehydrated, and embedded in paraffin. Samples were sectioned at 15 µm and mounted on charged glass slides, after which the paraffin was removed and the samples were rehydrated. Silver staining of samples was performed as described in Naoumenko and Feigin (1967).

*Acridine–orange staining:*

Imaginal discs were dissected in 1x PBS and incubated in a 1.6 x 10–6 M solution of acridine–orange solution for 10 min. Discs were rinsed and mounted in a 1x PBS solution.

RESULTS

**SD47M mitotic clones affect the development of bristles and legs:**

To gain a better understanding of the role that sd plays in development, we generated flip-in mitotic clones using an embryonic lethal allele of *sd*, $sd^{47M}$. The $sd^{47M}$ allele contains a 157-bp deletion spanning intron 8 and exon 9 of the gene (Srivastava et al., 2004). Because the deletion spans a splice site, it presumably disrupts the VID and the C-terminal region of the protein. The induction of $sd^{47M}$ mitotic clones causes defects in the wing (Liu et al., 2000), eye bristles, and the leg. Similar to the wing, clones in the eyes were very small. No *w, ry* clones were visible in the eye. In wild-type eyes, bristles are found at alternating vertices of each ommatidium (Figure 2.2, A and B). The effect of clonal induction on bristles varied. SEM analysis revealed that a few of the bristles were either smaller (Figure 2.2C) or mispatterned (Figure 2.2D). Wild-type legs are made up of three basic parts: the femur, the tibia, and the tarsus (Figure 2.2E). The distal end of the fly leg contains a metatarsal claw (Figure 2.2E, arrow and inset). Inducing *sd47M* clones leads to truncations in the leg (Figure 2.2F; arrow and inset emphasize the absence of the metatarsal claw).

**VGΔACT is able to antagonize SD function in the wing imaginal disc:**

In the wing, induction of mitotic clones causes only blistering, whereas homozygous viable mutant alleles of *sd* cause a loss of wing tissue. Because the clones are surrounded by a population of wild-type cells, it is possible that the loss of *sd* in the eye and leg is partially masked. To create a more robust phenotype, we created a construct, VGΔACT, which is able to bind to SD but is not able to activate transcription. The VGΔACT construct was generated by removing the two activation domains, ACT1 (amino acids 1–65) and ACT2 (amino acids 356–453) (Halder and Carroll, 2001; MacKay et al., 2003), from *vg* (Figure 2.1), leaving the entire scalloped interacting domain intact. Thus, the expectation is that the VGΔACT protein will cause a dominant-negative phenotype. To test if the VGΔACT construct can cause a dominant-negative effect, we first expressed it in the wing. Using the vgGAL4 driver, we expressed the VGΔACT transgene along the dorsal–ventral axis of the wing disc. This ectopic expression causes a loss of wing bristles and wing tissue (compare Figure 2.3B to Figure 2.3A). The extent of the phenotype varies considerably from almost a complete loss of the wing (not shown) to small patches of missing bristles in the anterior margin and loss of wing tissue along the posterior margin (Figure 2.3B). To test whether the observed effects are due to the VGΔACT protein antagonizing the endogenous SD protein, we attempted to rescue the dominant-negative phenotype by coexpressing full-length SD. Coexpression of full-length SD and VGΔACT rescued the bristle and the wing margin phenotypes (Figure 2.3C).

**VGΔACT is not able to antagonize SD function in the embryo:**

Since the VGΔACT protein is able to cause a dominant-negative phenotype in the wing, it may be able to have a similar effect in other tissues where SD is expressed. To test the ability of VGΔACT to antagonize endogenous SD in other cells, we expressed the transgene in the embryo using an actin GAL4 driver and a muscle-specific 24BGAL4 driver (Kidd et al., 1999). Overexpression of SD using the 24BGAL4 driver has been shown to cause embryonic lethality and to have an effect on the development of cardiac cells (Bidet et al., 2003). Furthermore, we found that *tinman*-expressing cells are mispatterned in a *sd* null background (data not shown). Neither embryonic lethality nor *tinman* mispatterning is seen when the VGΔACT or SD deletion transgenes are expressed under the control of either driver. However, larval lethality is seen when the transgenes are expressed using the *actin* GAL4 driver.

**VGΔACT is able to antagonize SD function in tissues outside the wing disc:**

Driving the VGΔACT transgene with the widely expressed ptcGAL4 driver causes more robust defects in the fly (Figure 2.4) than those seen with the $sd^{47M}$ mitotic clones. Antagonizing SD in the eye causes the eyes to protrude (compare Figure 2.4B to Figure 2.4A). We also observe more severe defects that affect bristle patterning, number, and morphology. In VGΔACT/+; *ptc* GAL4/+ flies, bristles can occur at adjacent vertices (Figure 2.4C; the ommatidium marked with an asterisk has bristles at four vertices) or they can be absent (Figure 2.4D, arrow). Duplications of bristles are also visible (Figure 2.4C, arrowhead) and in some of the more severe cases as many as three extra bristles at a

single vertex are seen (not shown). Expression of the VGΔACT protein also affects bristle size (Figure 2.4C; note the size differences between the bristles identified by the arrows). Interestingly, bristle defects are more frequent along the tip of the eye protrusion. In some eyes, defects in the ommatidia are also present. Changes in ommatidia vary from increases in size (data not shown) to fusions between adjacent ommatidia (Figure 2.4D; compare fused ommatidia marked by a single asterisk to wild-type ommatidia marked by a double asterisk).

Four structures make up the optic lobe: lobulla, lobulla plate, medulla, and lamina (Figure 2.4E) (Armstrong et al., 1995). In wild-type flies, the lamina makes direct contact with the basal membrane of ommatidia (Figure 2.4E). Silver staining of horizontal sections from VGΔACT/+; *ptc* GAL4 flies reveals an ectopic cluster of cells between the lamina and the basal membrane (Figure 2.4F, arrowhead) that correlates with the location of the protrusion (Figure 2.4F, arrow). All of the components of the optic lobe are present and appear normal in these flies. To try to identify the cells affected in the eye and brain, we expressed our construct with a variety of drivers (see Table 2.1). Only the eyeless GAL4 driver was able to cause optic lobe and eye phenotypes. None of the other eye- or brain-specific drivers had any effect.

Truncation can occur in all three leg segments when the VGΔACT transgene is under the control of the *Dll* GAL4 driver (Figure 2.4G; arrow and inset emphasize the absence of the metatarsal claw). The degree of leg truncation varies from loss of only a few of the metatarsals (Figure 2.4G, arrow) to the loss of all the metatarsals, tarsus, and tibia (not shown). Sharp bends in the femur or tibia are also sometimes seen (Figure 2.4G, arrowhead). In addition to causing truncations as seen in the mitotic clones, the VGΔACT protein is also able to cause duplications of the leg. However, only the VGΔACT/+; *ptc* GAL4 flies show duplications of the leg (Figure 2.4H, arrowhead) and these occur only in the T2 and T3 legs.

**SD likely binds to other cofactors:**

To help determine if the VGΔACT protein hinders the binding of important cofactors to SD, we expressed a truncated form of *sd* (SDΔ200) that lacks the coding capacity for the first 200 amino acids of SD (Figure 2.1). Although the SDΔ200 protein is missing the TEA/ATTS DNA-binding domain, it should still be able to bind to TIFs and compete with endogenous SD for binding to them. Thus, the expectation is that if SD binds to and interacts with other cofactors via a domain located in the terminal 244 amino acids of the protein, the SDΔ200 transgene should induce a phenotype similar to those seen in the VGΔACT/+; *ptc* GAL4 flies. If, however, SD does not interact with cofactors, or interacts with cofactors via a domain located within the first 200 amino acids of the protein, the SDΔ200 transgene should have no effect. Expression of the SDΔ200 protein with the *ptc* GAL4 driver causes phenotypes similar to those observed with the VGΔACT construct (Figure 2.5). In the head, the SDΔ200 protein causes an eye protrusion (data not shown), alters bristle patterning (Figure 2.5A, arrow) and morphology (Figure 2.5B, arrow), causes duplication of bristles (Figure 2.5A, arrowhead), induces the fusion of ommatidia (Figure 2.5C, asterisk), and produces an ectopic cluster of cells between the

lamina and ommatidia (Figure 2.5D). SDΔ200 is also able to cause duplications in the legs (Figure 2.5E) and a loss of wing bristles and tissue (Figure 2.5F).

**SD likely functions as a TF in tissues outside the wing disc:**

To determine if the sole function of SD in larval tissues is to transport proteins into the nucleus, we overexpressed SDΔ88-123 and SDΔ88-159 (Figure 2.1) using the *ptc* GAL4 driver. The protein encoded by the SDΔ88-123 transgene contains a deletion in the first half of the TEA/ATTS DNA-binding domain that does not compromise the nuclear localization signal (NLS), while the deletion in the SDΔ88-159 removes the entire TEA/ATTS DNA-binding domain and the majority of the NLS. If the sole function of SD is to transport proteins into the nucleus, expression of the SDΔ88-159 but not the SDΔ88-123 transgene should be able to induce phenotypes similar to those observed by expressing VGΔACT and SDΔ200. Expression of either the SDΔ88-123 or the SDΔ88-159 proteins is able to induce similar eye, brain, wing, and leg phenotypes (data not shown), indicating that the role of SD in these tissues is not limited to transporting proteins into the nucleus.

**Levels of SD are important in tissues outside the wing disc:**

To assess whether the relative levels of SD are as important for the proper development of the eye, brain, and leg as they are in the wing (Halder et al., 1998; Simmonds et al., 1998), we overexpressed the full-length SD transgene (Figure 2.1) using the *ptc* GAL4 driver. Unfortunately, this caused pupal lethality. Removal of pharate adults from the pupal cases reveals phenotypes similar to those seen in the presence of the VGΔACT and the SDΔ200 proteins. Protrusion of the eyes (Figure 2.6A, arrowhead), truncations of the legs (Figure 2.6B, arrowheads), and an ectopic cluster of cells below the basal membrane of the ommatidia (Figure 2.6C) are all seen. In most of the pupae, the head of the fly is found in the abdomen (Figure 2.6D). Silver staining of the horizontal sections in these flies shows that the lamina and regions of the optic lobe are missing (Figure 2.6, C and E). The brain is also physically separated from the optic lobe (Figure 2.6E). However, the development of the ommatidia remains intact (Figure 2.6F).

**SD is required for cell survival in the leg disc:**

One function of the SD/VG complex in the wing is to promote cell survival (Delanoue et al., 2004; Liu et al., 2000). To ascertain if SD also plays a role in promoting cell survival in other larval tissues, we looked for the presence of increased cell death in our transgenic flies using acridine–orange staining. In the wild-type wing disc, SD expression is restricted to the wing pouch and the periphery of the notum. Very little to no cell death is visible in the wing (Figure 2.7A) and leg (Figure 2.7B) imaginal discs of the wild-type fly. Staining of *ptc* GAL4; UAS SDΔ200 (Figure 2.7C, arrow) and *ptc* GAL4; UAS VGΔACT (data not shown) imaginal discs shows only increased acridine–orange staining along the anterior/posterior (A/P) boundary of the wing pouch. No increase in staining is seen along the A/P boundary of the notum (Figure 2.7C, arrowhead). Increased staining is also seen in the leg imaginal disc (Figure 2.7D). In addition to an increase in staining in the leg discs, the leg discs may also be duplicated (Figure 2.7D). No notable change in

acridine staining is visible in the larval optic lobes or in the eye-antennal imaginal disc (data not shown).

## DISCUSSION

The generation of $sd^{47M}$ mitotic clones resulted in a mild phenotype in the eye and leg and had no effect in the optic lobe. Considering that induction of $sd^{47M}$ mitotic clones also caused a weak blistering phenotype in the wing (Liu et al., 2000), it is not surprising that they had a minor effect in tissues outside the wing. The weak phenotype is due to the inability of the clones to survive. No *w, ry* clones were seen in the eye, despite the fact that numerous blisters are present in the wing. However, the induction of clones is still able to cause a bristle phenotype, indicating that SD may be required for the survival of some of the eye cells.

To create a stronger phenotype, we attempted to antagonize the SD protein in the developing embryo and larva. This was accomplished by ectopically expressing a truncated form of VG, VGΔACT, which can still bind to SD but cannot activate transcription of downstream genes. Thus, we hoped that the interaction between the VGΔACT protein and SD would create an inert complex and consequently affect overall SD function. To test if the VGΔACT protein can antagonize SD function, we expressed it in the wing. Similar to *sd* mutant flies, the VGΔACT protein in the wing is able to induce wing-pouch-specific cell death (Figure 2.7C) (Liu et al., 2000) and to promote the loss of bristles and wing tissue (Figure 2.3B) (Campbell et al., 1992; Simmonds et al., 1998). These observations indicate that the VGΔACT protein likely binds to SD and disrupts its function in the wing.

While ectopic expression of full-length SD using either the *actin* or the 24BGal4 drivers causes embryonic lethality, the VGΔACT and the SD deletion proteins are unable to affect embryonic development. Several possibilities may explain why the VGΔACT protein is unable to disrupt SD function in the embryo: (1) proteins with which SD interacts in the embryo may utilize a domain that the VGΔACT protein is unable to affect or may bind to a region outside of the SDΔ200 protein, (2) the TIFs with which SD interacts in the embryo have a higher binding affinity than the VGΔACT protein, or (3) SD may have a TIF-independent role in the embryo. The inability of our construct to inhibit SD function in the embryo is consistent with the observation that lethal alleles of *sd* carrying a point mutation in either the VID or the putative TD do not cause embryonic lethality (Srivastava et al., 2004), but do cause pupal lethality. Insight into the regions of SD required for embryonic development may come from the fact that the SD human homolog TEF-1 is able rescue the embryonic lethal allele $sd^{ETX81}$ (Deshpande et al., 1997). These observations, taken together, indicate that the mechanism by which SD functions in the embryo is likely different from that in larval tissues.

The VGΔACT protein, however, is able to antagonize SD function in larval tissues. Ectopic expression of the protein is able to disrupt leg, eye, and brain development. In the leg, expression of the VGΔACT transgene is able to induce duplications and truncations (Figure 2.4, G and H). Several lines of evidence indicate that the phenotypes seen in the leg may be related to an induction of cell death, rather than to a defect in

pattern formation. Previous reports have shown that high levels of apoptosis induced by ultraviolet (UV) light in the leg are able to induce splitting and duplication of the T2 and T3 but not the T1 appendage (Arking, 1974). Similar to the UV experiments, duplications caused by the VGΔACT protein in the leg disc are seen only in the second and third appendages. The lack of duplications seen in the T1 segment supports the idea that the phenomenon is related to cell death. Furthermore, dissections of VGΔACT/+; *ptc* GAL4/+ larvae show splitting of the leg imaginal disc and an increased level of cell death (Figure 2.7D). Finally, expression of the construct at the distal end of the leg with the DllGAL4 driver induces truncations at the tip of the appendage (Figure 2.4G).

Antagonizing the SD protein in the eye affects proper development of the bristles and ommatidia. Defects in bristles include changes in morphology and patterning. Previous reports have shown that SD is able to activate genes involved in sensory organ development (Halder et al., 1998; Srivastava and Bell, 2003) and expression of the *sd* TEA::VG fusion construct in the eye is able to alter bristle morphology (Srivastava et al., 2002). Thus, it is likely that SD has a role in patterning eye bristles. Another possible explanation for the bristle defects may be related to the cone cell phenotype. Pigment cell identity in the developing eye is based on cues provided by precursor cone cells (Cagan and Ready, 1989a, b). The secondary and tertiary pigments can be sacrificed to form extra bristle cells (Cagan and Ready, 1989a, b). Thus, it is possible that the bristle phenotype may be due to defects in cone cell patterning. The ommatidial defects are seen on the surface of the fly eye and not in the horizontal sections. The inability to see these effects in the horizontal sections may be because ommatidial fusions occur at a low frequency in the eye (typically four to five fused ommatidia are seen in each eye) or because of cone-cell-patterning defects. Expression of the VGΔACT protein is also able to cause a protrusion in the adult eye (Figure 2.4B). Horizontal sections show that the protrusion is due to an unidentified ectopic layer of cells (Figure 2.4F). These cells are located between the lamina and the ommatidia (Figure 2.4F, arrowhead). Overexpression of SD induces similar, but more severe, phenotypes than when the VGΔACT or SDΔ200 proteins are expressed (Figure 2.6). In flies where SD is overexpressed, components of the optic lobe are lost (Figure 2.6, C and E). Thus, it is possible that the ectopic layer of cells originates from the optic lobe.

To determine if SD forms a TF complex in larval tissues, we expressed a truncated form of SD, SDΔ200 (Figure 2.1). The DNA-binding domain of SD and the NLS are deleted in the SDΔ200 transgene. However, the SDΔ200 protein should still bind to and potentially sequester SD-specific TIFs. Expression of the SDΔ200 protein (Figure 2.5) is able to induce phenotypes similar to those seen with the VGΔACT protein, suggesting that SD probably binds to cofactors in the eye, leg, and brain. In larval tissues, SD could interact with a putative TIF via the VID or perhaps even the TD. The existence of a TD domain in SD is supported by the observation that a mutation in the C-terminal end of the gene causes pupal lethality, but does not inhibit wing development (Srivastava et al., 2004). However, whether or not this association forms a TF complex is not clear. In the wing, SD is required to transport VG into the nucleus (Halder et al., 1998; Simmonds et al., 1998; Srivastava et al., 2002). Thus, it seemed possible that the sole function of SD in the leg, eye, and optic lobe would be to transport proteins into the nucleus. Expression

of a truncated form of SD that contains an intact NLS, but is unable to bind to DNA, is still able to induce all the mutant phenotypes, indicating that SD function in larval tissues is not restricted to transporting proteins into the nucleus.

In other species, whenever a TEAD protein interacts with a TIF it forms a TF complex. In mammals, the TEAD protein (TEF-1) is known to interact with a variety of different TIFs, such as VGL-1 and VGL-3 in the placenta (Halder et al., 1998; Maeda et al., 2002), VGL-2 in skeletal muscles (Maeda et al., 2002), VGL-4 in the heart (Chen et al., 2004b), YAP65/Yorkie (Vassilev et al., 2001; Wu et al., 2008; Zhang et al., 2008), and p160 (Belandia and Parker, 2000). In each case, the interaction between TEF-1 and its relevant TIF results in a TF complex. Thus, it is likely that in larval tissues the association between SD and putative TIFs also results in the formation of a TF complex (SD/TIF). The identity of putative TIFs with which SD may interact is currently unknown. Homology searches reveal three potential novel interacting partners for SD. The VGL-4 homolog in Drosophila is an unidentified gene, CG10741. To date there have been no studies done with CG10741. The p130 homolog, *taiman*, is widely expressed in the embryo and follicle cells in the larva (Bai et al., 2000).

One interesting property of SD is that its amount is important for proper wing development (Simmonds et al., 1998). A change in the ratio of SD to VG affects the development of the wing. Overexpressing SD causes phenotypes in leg, eye, and brain similar to those observed when the SD/TIF complex is antagonized. Why the levels of SD are important for its proper function remains unclear. In the wing, the SD/VG complex requires two SD molecules and two VG molecules (Halder and Carroll, 2001). SD can form homo-dimers (Halder et al., 1998) and thus the overexpression of SD may promote the formation of SD dimers as opposed to forming a tetrameric TF complex with its TIF. Another possibility may be that free SD may act as a repressor (Anbanandam et al., 2006) and that overexpressing SD may lead to suppression of genes that are required to promote cell survival in the brain, eye, and leg.

When SD function is disrupted in the leg and the eye, a common phenotype observed is the loss of tissue. Bristles are lost in the eye (Figure 2.4D) and truncation can be seen in the legs (Figure 2.4G). Furthermore, the induction of clones in the eye did not give rise to any *w, ry* clones. In the wing, SD has a crucial role in promoting cell survival (Delanoue et al., 2004; Liu et al., 2000) and this role may also be important in the eye and the leg. Acridine–orange staining of leg discs where SD is antagonized shows an increased amount of cell death (Figure 2.7D), suggesting that the complex is required to promote cell survival. Whether this is a direct relationship is currently unclear. The increased cell death may be because the cells in these tissues fail to properly differentiate, and consequently are unable to interpret the proper cues to survive. No increase in acridine–orange staining was observed in the eye disc (not shown). There is a high level of background acridine–orange staining (data not shown) and missing bristles occur only in a small percentage of the ommatidia, but this does not exclude the possibility that the SD/TIF complex is required to promote cell survival in these tissues.

Determining the role that SD has in promoting the fate of eye cells, leg cells, or optic lobe cells is difficult without knowing the identity of the possible TIFs with which it associates. Thus, the exact role for such a complex in these tissues must still be determined. However, we were able to gain some insight into the function of SD in these tissues by generating mitotic clones and expressing a truncated form of VG, VGΔACT. The VGΔACT protein is able to bind to SD and likely prevents TIFs from interacting with SD protein:protein interaction domains that include any of the amino acids between positions 220 and 344 (Vaudin et al., 1999). In the wing, the SD/VG complex is required to determine wing fate (Halder et al., 1998) and to promote cell survival (Delanoue et al., 2004; Liu et al., 2000). We have shown that the mechanism by which SD functions in the eye, leg, and brain is similar to that of SD in the wing imaginal disc. SD likely binds to tissue-specific TIFs to form a TF and this interaction occurs by a domain located in the C-terminal portion of the protein. The TF complex is also sensitive to the levels of SD present in the system and antagonizing SD function induces cell death. Thus, the SD/TIF complexes in larval tissues may be involved in promoting optic lobe, bristle, ommatidia, and leg fate or be involved in regulating cell proliferation and/or promoting cell survival. Studies aimed at identifying the TIFs or at determining the exact protein:protein interaction domains that these TIFs recognize may be able to identify the role of SD in these tissues.

TABLE 2.1 Eye and neural drivers used to express VGΔACT and SDΔ200

| Driver | Expression pattern | Phenotype |
|---|---|---|
| Actin Gal4 | Ubiquitous | Larval lethal |
| Ato Gal4 | In ato+ cells in the brain and SOP | No effect |
| Cha Gal4 | In all cholinergic neurons | No effect |
| CQ2Gal4 | In U/CQ neurons | No effect |
| Dll GAL4 | Distal region of the leg disc | Distal truncation of legs |
| ey GAL 4 | ey+ cells in the eye disc and the larval brain | Protrusion of the eyes. |
| | | Bristle defects and fusion of ommatidia. |
| GMR Gal4 | GMR+ cells in the eye disc | No effect |
| Pan-R7Gal4 | In all R7 cells | No effect |
| PdfGal4 | In ventrolateral neurons of the brain | No effect |
| RN2Gal4 | In RP2, aCC and pCC neurons | No effect |
| sevEPGal4 | In sev+ cells | No effect |
| 24BGal4 | Embryonic mesoderm | No effect |
| 109(2)80Gal4 | Dendritic neurons | No effect |
| 167YGal4 | Neuroblast in the central brain and ventral ganglion | No effect |
| 179YGal4 | Outer proliferative center near central brain | No effect |
| C147Gal4 | Larval brain | No effect |
| c698aGal4 | In the third instar larval brain | No effect |

FIGURE 2.1. Schematic of the VG and SD constructs used herein. Within the 453 amino acid (aa) VG protein, two putative activation domains, ACT-1 (aa 1–65) and ACT-2 (aa 335–453), and a Scalloped-interacting domain, SID (aa 281–335), have been identified. The two activation domains are removed in the VGΔACT construct. The 440-aa SD protein contains a TEA/ATTS DNA-binding domain, TEAD (aa 88–159); a Vestigial-interacting domain, VID (aa 220–344); and an NLS (aa 144–162). The TEAD is no longer functional in the SDΔ88-123 construct while the TEAD and the NLS are nonfunctional in the SDΔ88-159 and SDΔ200 constructs.

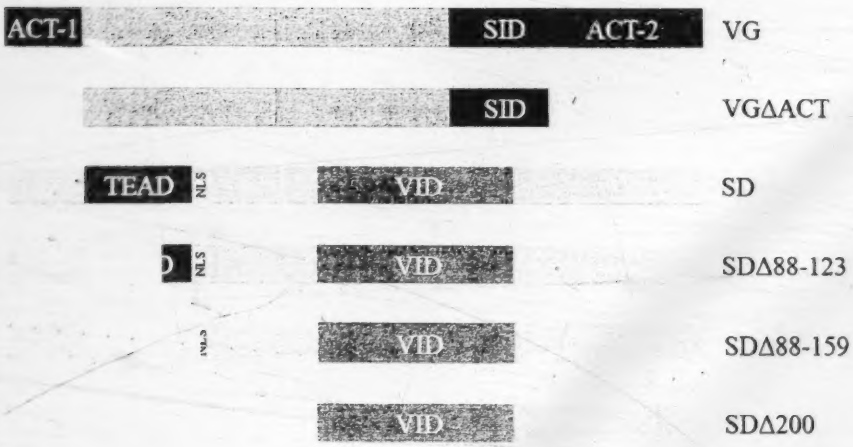
ACT-1
SID
ACT-2
VG
SID
VGΔACT
TEAD
NLS
VID
SD
NLS
VID
SDΔ88-123
VID
SDΔ88-159
VID
SDΔ200

FIGURE 2.2. Mitotic clones of $sd^{47M}$ cause defects in the eye and leg. (A) Wild-type ommatidia (x2000). (B) Illustration of normal bristle patterning in the eye. Hexagons represent individual ommatidia and circles represent bristles. In wild-type flies, bristles occur at alternating vertices of the ommatidium. (C) Bristles are sometimes shorter (asterisk) in $sd^{47M}$ mitotic clones. (D) Duplications and mispatterning (asterisk) of bristles also occurs in $sd^{47M}$ mitotic clones. (E) Wild-type T1 leg. Arrow indicates the metatarsal claw shown in the inset. The femur (fem), tibia (tib), and tarsus (tar) are marked. (F) Leg tissue and the metatarsal claw is lost (arrow and inset) in $sd^{47M}$ mitotic clones.

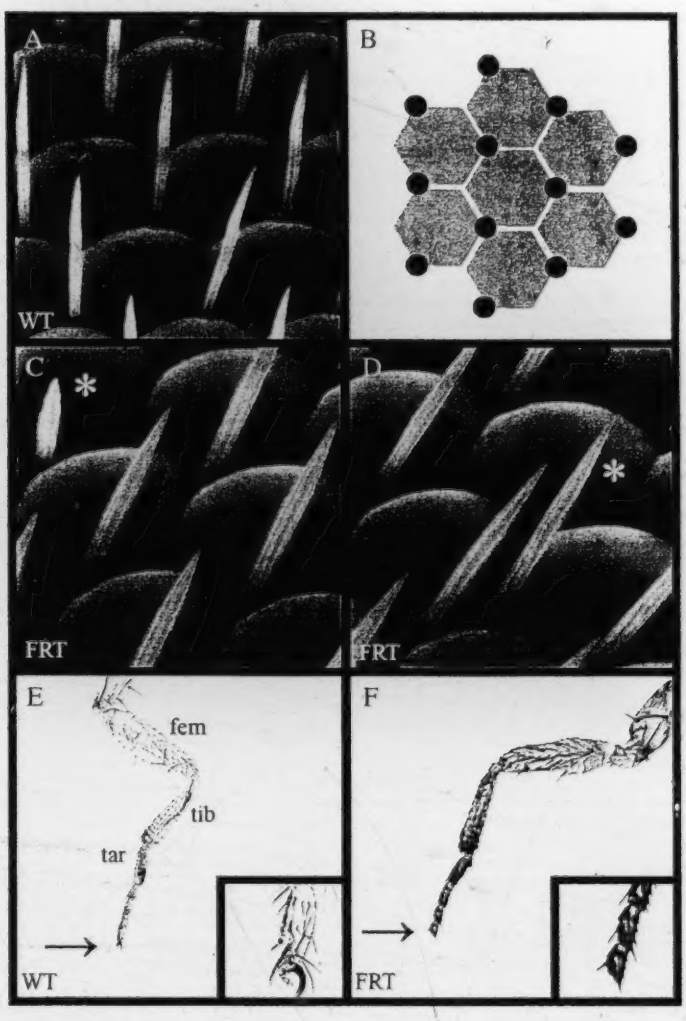
A
WT
B
C
*
FRT
D
*
FRT
E
fem
tib
tar
WT
F
FRT

FIGURE 2.3. VGΔACT interacts with SD and disrupts SD function. (A) A wild-type wing. (B) Wing bristles and wing tissue are lost in a *vg*GAL4/+; UAS VGΔACT/+ fly. (C) Rescue of the *vg*GAL4/+; UAS VGΔACT/+ phenotype by coexpression of full-length SD.

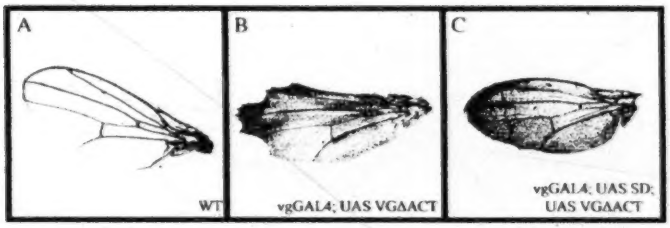
A
WT
B
vgGAL4; UAS VGΔACT
C
vgGAL4; UAS SD;
UAS VGΔACT

FIGURE 2.4. Antagonizing SD in tissues outside the wing. (A) Wild-type eye (x200). (B) Eyes protrude (arrowhead) in a *ptc*GAL4/+; UAS VGΔACT/+ fly (x200). (C and D) Bristle pattern is affected in a *ptc*GAL4/+; UAS VGΔACT/+ fly (x2000). (C) Bristles are duplicated (arrowhead), and the size of the bristles is affected (arrows). Patterning defects are seen in the ommatidium marked by an asterisk. Four vertices, rather than three, of this ommatidium have bristles. (D) Bristles can also be missing (arrow) and ommatidia may be fused [compare the fused ommatidia (single asterisk) to the normal ommatidium (double asterisk)]. (E) A silver-stained horizontal section of a wild-type eye. The structures of the eye are labeled: lo, lobula; lo p, lobula plate; me, medulla; lam, lamina; and om, ommatidia. (F) An ectopic cluster of cells is present between the lamina and the ommatidia (arrowhead) in a *ptc*GAL4/+; UAS VGΔACT/+ fly eye. The ectopic cluster of cells coincides with the protrusion in the eye (arrow). (G) Leg tissue and the metatarsal claw is lost (arrow and inset) in a *Dll*GAL4/+; UAS VGΔACT/+ fly. Legs may also contain a kink within the tibia (arrowhead). (H) Single leg showing that it has been duplicated at the base of the joint in a *ptc*GAL4/+; UAS VGΔACT/+ fly (arrowhead). The duplicated structures are also truncated.

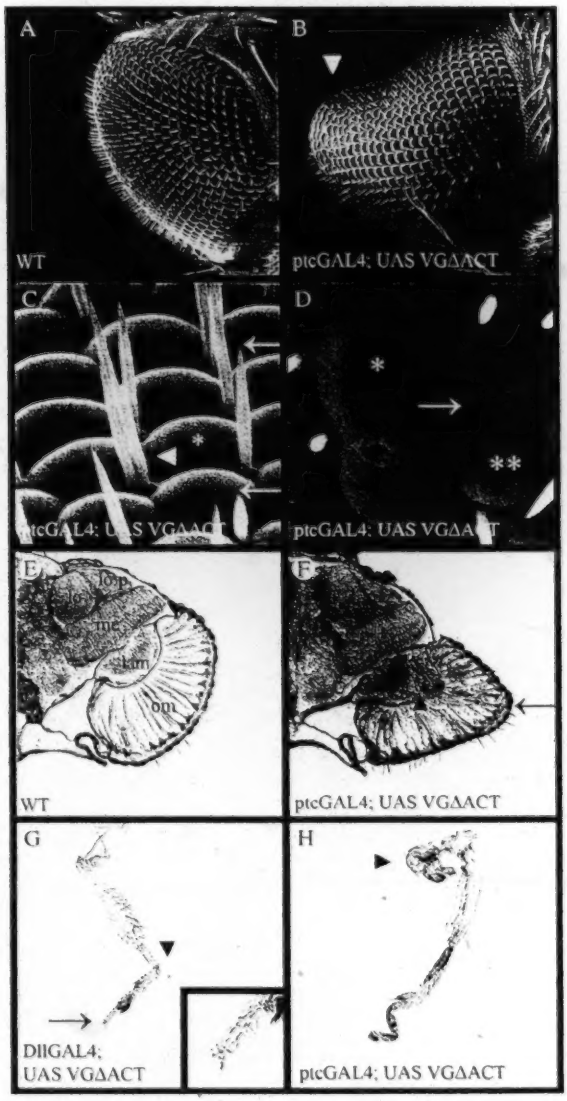
A
WT
B
ptcGAL4; UAS VGΔACT
C
D
ptcGAL4; UAS VGΔACT
E
lop
me
lam
om
WT
F
ptcGAL4; UAS VGΔACT
G
DllGAL4;
UAS VGΔACT
H
ptcGAL4; UAS VGΔACT

FIGURE 2.5. The SDΔ200 protein can also antagonize SD function. *ptc*GAL4/+; UAS SDΔ200/+ flies (x2000) have mutant phenotypes similar to *ptc*GAL4/+; UAS VGΔACT/+ flies. (A) Bristles are duplicated (arrowhead) or missing (arrow). Also note that several of the ommatidia have bristles at four of their vertices. (B) Alterations in bristle size are common (arrow). (C) Fusion of ommatidia (fused ommatidia is marked by asterisk). (D) A silver-stained horizontal section shows an ectopic cluster of cells (arrowhead) that coincides with the eye protrusion (arrow). (E) The legs are occasionally duplicated. (F) Wing tissue and bristles are also lost in a *vg*GAL4/+; UAS SDΔ200/+ fly.

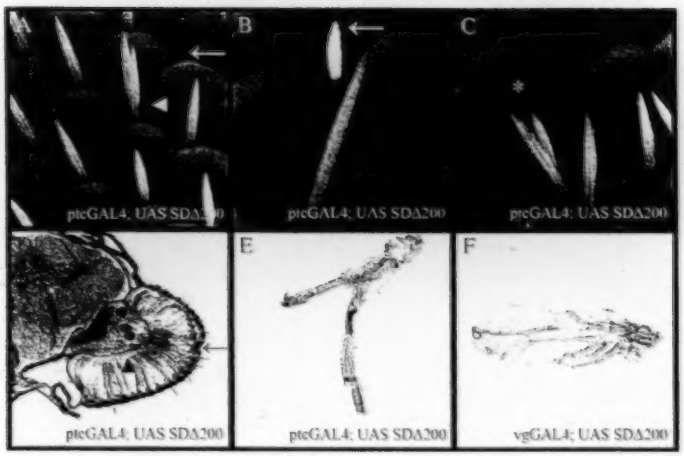
A
B
C
ptcGAL4; UAS SDΔ200
ptcGAL4; UAS SDΔ200
ptcGAL4; UAS SDΔ200
ptcGAL4; UAS SDΔ200
E
ptcGAL4; UAS SDΔ200
F
vgGAL4; UAS SDΔ200

FIGURE 2.6. SD levels are important for proper development. Overexpression of wild-type SD in *ptc*GAL4/+; UAS SD/+ flies causes (A) eye protrusions (arrowhead) and (B) truncated legs (arrowheads). Overexpression of SD does not affect (C) the lobula (lo), medulla (la), or ommatidia (om), but does affect the lobula plate and lamina as these structures are missing. An ectopic cluster of cells is also present (arrowhead). In an extreme situation (D), the head remains inside the abdominal cavity (arrowhead). Silver staining of a horizontal cross section shows (E) that the brain (br) and the medulla (me) are located in the abdominal cavity and are no longer connected. The lamina and lobula are absent. The structure of the ommatidia (F) is preserved.

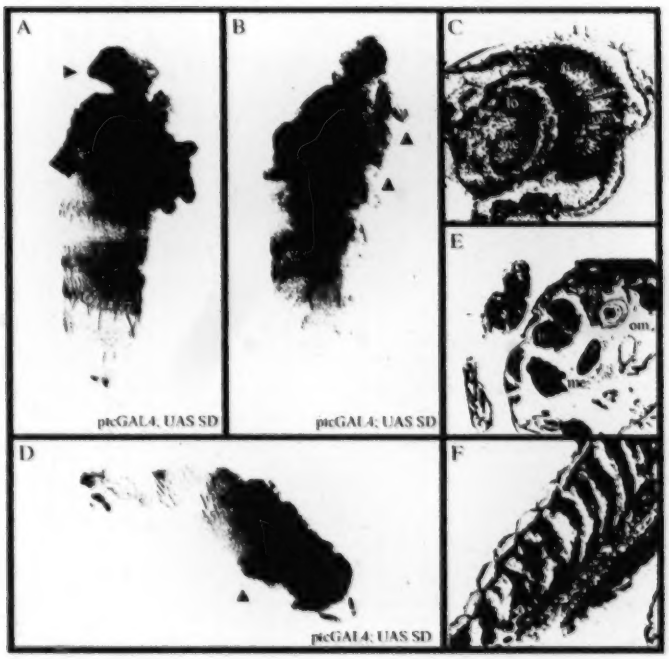
A
B
C
E
D
F
ptcGAL4, UAS SD
ptcGAL4; UAS SD
ptcGAL4; UAS SD
lo
om

FIGURE 2.7. SD promotes cell survival in the leg imaginal disc. Acridine–orange staining in a (A) wild-type wing imaginal disc, (B) wild-type leg imaginal disc, (C) *ptc*GAL4; UAS SDΔ200 wing imaginal disc, and (D) *ptc*GAL4; UAS SDΔ200 leg imaginal disc. Very little cell death is visible in the wild-type wing and leg imaginal discs. Ectopic expression of SDΔ200 induces cell death only in the (C) wing pouch (arrow) and not in the notum (arrowhead). Increased cell death is also visible in the (D) leg imaginal disc.

A
B
C
D
WT
WT
pleGAL4; UAS SDΔ200
pleGAL4; UAS SDΔ200

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## CHAPTER III - Nerfin-1: A novel binding partner of Scalloped.[2]

### INTRODUCTION

The TEA/ATTS DNA binding domain (TEAD) defines a highly conserved family of transcription factors that recognizes the M-CAT motif (5'TCATTCCTT-3') (Hwang et al., 1993; Stewart et al., 1994). Because TEA/ATTS domain proteins generally lack an activation domain (Hwang et al., 1993; Laloux et al., 1994; Vaudin et al., 1999; Xiao et al., 1991), they associate with transcriptional intermediary factors (TIFs) (Belandia and Parker, 2000; Butler and Ordahl, 1999; Chen et al., 2004; Gupta et al., 2001; Gupta et al., 1997; Halder et al., 1998; Maeda et al., 2002; Simmonds et al., 1998; Vassilev et al., 2001) in order to properly function as a transcriptional complex. TEAD proteins are expressed in a wide variety of cells and can bind to tissue specific and non-tissue specific TIFs. Several of these binding partners are known. TEAD proteins have been shown to associate with the nuclear protein poly(ADP- ribose) polymerase (Butler and Ordahl, 1999), the p160 family of transcription factors (Belandia and Parker, 2000), the basic-helix-loop leucine zipper protein Max (Gupta et al., 1997), YAP65 (Vassilev et al., 2001) , and the serum response factor (SRF) (Gupta et al., 2001) in a non-tissue specific manner and to vestigial (Halder et al., 1998; Simmonds et al., 1998) and vestigial-like proteins (Chen et al., 2004; Maeda et al., 2002) in a tissue specific manner. Work with these binding partners reveals that these TIFs can interact with the TEAD proteins through the second and third helices of the TEA/ATTS DNA binding domain (Gupta et al., 2001), the C-terminal protein: protein interaction domain or the vestigial interaction domain (VID) (Chen et al., 2004; Maeda et al., 2002; Vassilev et al., 2001; Vaudin et al., 1999). However, despite the numerous TIFs that have been identified very little is known about the domains these cofactors use to interact with TEAD proteins.

The *Drosophila melanogaster* genome contains a single TEAD-encoding gene, *scalloped* (*sd*), which is expressed in the nervous system, wing and eye imaginal discs, as well as the optic lobe (Campbell et al., 1992). In the wing imaginal disc, SD interacts with its tissue specific TIF, Vestigial (VG) (Halder et al., 1998; Simmonds et al., 1998). Together, these two proteins specifically activate downstream wing genes (Halder et al., 1998; Simmonds et al., 1998) and promote wing fate differentiation (Kim et al., 1997). Mutations in these genes cause a dramatic loss of wing tissue (Campbell et al., 1992; Campbell et al., 1991; Williams et al., 1991; Williams et al., 1993). In vitro binding experiments have identified a 54 amino acid SD binding domain (Simmonds et al., 1998) referred to as the scalloped interaction domain (SID). The identification of SID prompted the discovery of the human vestigial-like proteins (VGL) (Chen et al., 2004; Maeda et al., 2002; Vaudin et al., 1999) with a similar motif. Alignment of the SID from the VG and VGL proteins reveals a consensus $V^{E}/_{D}{}^{E}/_{D}$HFRALG motif that is probably essential for its interaction with the VID (Chen et al., 2004).

Over-expression studies of just the SID or the VID, in *D. melanogaster* larval tissues, indicate that SD likely interacts with additional proteins besides vestigial (Garg et al.,

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[2] The *sd* deletion constructs used in Fig 3.3 were created by Hua Deng
The anti-Fas2 antibody staining was performed by Thomas Brody

2007). A yeast-2-hybrid study pulled out two novel Drosophila cofactors, Yorkie and an EIN domain containing transcription factor, Nervous Finger-1 (Nerfin-1) (Giot et al., 2003). Both Yorkie and the human homolog of Yorkie, Yap65, have been shown to interact with SD and the human TEAD proteins (Vassilev et al., 2001), respectively. Despite the fact that the *C. elegans* TEAD containing protein, EGL- 44, is able to activate the expression of the EIN domain coding gene *egl-46* (Wu et al., 2001), EIN domain proteins have not been shown to directly interact with TEAD proteins. In *C. elegans*, EGL-44 activates the expression of *egl-46,* in FLP cells (Wu et al., 2001). *egl-46* is present in both touch and FLP neurons, but *elg-44* expression is restricted to FLP cells. The presence of both EGL-44 and EGL-46 is able to suppress touch cell differentiation and promote a FLP fate (Wu et al., 2001). The *egl-46* gene is also expressed in HSN cells, where it is required for the proper migration of the neuron (Wu et al., 2001). Mutations in *Drosophila* EIN protein affect early neuronal migration patterns (Kuzin et al., 2005).

Using both *in vivo* and *in vitro* experiments, we are able to show that Nerfin-1 can interact with SD. Like VG, Nerfin-1 recognizes and binds to the VID of SD. We were also able to localize the domain that Nerfin-1 uses to interact with SD to a 21 amino acid sequence that has a similar amino acid composition as the region that VG uses to interact with SD.

## MATERIALS AND METHODS

*Generation of Constructs*- The Nerfin-1 3' deletion constructs were generated from a UAS *Nerfin-1* template. The PCR products were digested with *Kpn*I and *Eco*RI and cloned into a pMT/V5 HIS B vector (Invitrogen). The internal Nerfin-1 deletions were generated by inverse PCR. The inverse PCR products were ligated with Promega T4 DNA ligase. (see Appendix I for a list of the primers used)

*Cell Culture and Transfection – Drosophila* S2 cells were grown in CCM3® (Hyclone) at 21 $^0$C. Transfections were carried out 24 hrs after plating, using *Cellfectin*®reagent according to the manufacturer's instructions (Invitrogen).

*Co-immunoprecipitation and Immunoblotting* – At 24 hrs post transfection, expression of the constructs was induced through either the heat shock promoter or the metallothionein (PMT) promoter by adding Cadmium Chloride to a final concentration of 10 µM (Prosise et al., 2004). At 48 hrs after induction, cells were harvested and lysed. Cells intended for the FLAG TAG pull down experiments were lysed using a 20 mM Hepes, 50 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM DTT, 1 % Triton-X lysis buffer (Hughes and Fehon, 2006), while those being used for a 6 X HIS TAG pull down used a 50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, 1% Glycerol lysis buffer. The cell lysate was incubated on a shaking incubator at 4$^0$C overnight with either 20 µM ANTI-FLAG M2 Agarose Beads (Sigma A2220) or 20 µM of 5% Ni NTA Magnetic Beads (QIAGEN). The ANTI-FLAG M2 agarose beads were washed 4X with the lysis buffer, while the 5% Ni NTA Magnetic Beads were washed 4X with a 50 mM $NaH_2PO_4$, 200 mM NaCl, 20 mM imidazole, 1% Glycerol wash buffer. Proteins were eluted from the agarose beads by boiling at 80 $^0$C for 10 mins with a 5X loading buffer (0.313 M Tris-

HCl, 10% SDS, 0.05% bromophenol blue, 2M DTT, 50% glycerol). Proteins were eluted from the 5% Ni-NTA Beads with a 20 mM Tris, 50 mM NaCl, 400 mM imidazole, 1% Glycerol elution buffer mixed with a 5X loading buffer. Supernatants were run on a standard 8% acrylamide gel. Full length Nerfin-1 protein was visualized using a primary guinea pig anti-Nerfin-1 (1:2500) (Stivers et al., 2000) and a secondary peroxidase-conjugated AffiniPure Goat Anti Guinea Pig IgG (H+L) (1:2500; Jackson immuneresearch), while the SD and SD deletion proteins were stained with a mouse anti-FLAG (1:1000; Sigma) primary antibody and a goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (1:1000; Invitrogen) secondary Ab. The membranes were incubated with SuperSignal® West pico Chemiluminescent substrate (PIERCE) and analyzed by autoradiography.

LacZ staining

Third instar $sd^{ETX4}$ larvae were collected and dissected. Imaginal discs were removed and fixed in 0.75% glutaraldehyde in 1XPBS for 20 min. Imaginal discs were washed in 1XPBT and incubated for 8 hours at $37^0$C in a staining solution (10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.2% X-gal). Imaginal discs were again washed in a 1XPBT solution, mounted and photographed.

RESULTS

**Nerfin-1 and SD expression patterns overlap in the third instar eye imaginal disc and optic lobe**

The $sd^{ETX4}$ allele has a P-element, containing a *lacZ* gene, inserted upstream of the *sd* transcription start site (Anand et al., 1990). This *lacZ* gene is expressed in a pattern similar to *sd* (Campbell et al., 1992). Lac Z staining in third instar larvae shows that *sd* is transcribed in the distal tip of the leg disc (Figure 3.1A), the pouch cells of the wing imaginal disc (Figure 3.1B), cells posterior to the morphogenic furrow in the eye imaginal disc (Figure 3.1C), and in the optic lobe (Figure 3.1D). Nerfin-1 Gal4; UAS GFP flies show that the Nerfin-1 gene is only expressed in the eye imaginal disc and the optic lobe (Figure 3.1E and 1F). No Nerfin-1 expression is detectable in the wing imaginal disc (data not shown). In the eye imaginal disc, *sd* and Nerfin-1 appear to be present in the same cells. In the optic lobe, Nerfin-1 is expressed in a sub set of *sd* expressing cells. These cells were previously identified as lamina precursor cells (Stivers et al., 2000).

**Nerfin-1 interacts with SD *in vivo* and *in vitro***

Mutations or deletions in either the SID of VG or the VID of SD, respectively, lead to a loss of wing tissue (Srivastava et al., 2004) or the inability to rescue a mutant wing (Chow et al., 2004; MacKay et al., 2003). Furthermore, ectopic expression of a protein that can compete with normal VG for its binding partner also leads to a loss of wing tissue (Garg et al., 2007). Directing expression of the VGΔACT protein, a truncated form of VG that can bind to SD but cannot activate transcription, along the dorsal-ventral

axis of the wing blade causes scalloping of the wing (compare Figure 3.2A to Figure 3.2B). The levels of SD protein can be artificially increased by adding an ectopic copy of the *sd* cDNA. Increasing the amount of SD protein can rescue the VGΔACT dominant negative phenotype (Figure 3.2C). To test if Nerfin-1 can interact with SD, we ectopically expressed Nerfin-1 in the wing imaginal disc along the dorsal ventral axis. The presence of Nerfin-1 in the wing blade resulted in a loss of wing tissue (Figure 3.2D). Similar to the VGΔACT experiment, the phenotype caused by the ectopic expression of Nerfin-1 can be rescued by artificially raising the levels of SD (Figure 3.2E). Raising the levels of SD can only rescue the wing blade phenotype when an ectopic protein is hindering the binding of SD to its cofactor VG, since just raising the levels of SD in a wild type background also causes a loss of wing tissue (Figure 3.2F).

To determine if SD and Nerfin-1 interact *in vitro*, co-immunoprecipitation experiments were performed. S2 cells were transfected with the Nerfin-1 cDNA and either an N-terminal FLAG tagged SD cDNA or an untagged SD cDNA. After 48 hours, the cells were lysed and incubated with flag antibody conjugated agarose beads, washed, run on an SDS/PAGE gel and analyzed by a Western blot. The presence or absence of the Nerfin-1 protein was confirmed by using an anti-goat Nerfin-1 antibody. Only the lane containing lysate from the S2 cells transfected with the Nerfin-1 and the N-terminal Flag tagged SD genes showed a band on the Western blot, indicating that SD and Nerfin-1 proteins are able to interact with each other. Lanes containing lysate from cells transfected with either Flag tagged SD or Nerfin-1, or with untagged SD and Nerfin-1 did not show a band on the Western blot (Figure 3.3A).

**The Vestigial Interaction Domain is used to Interact with Nerfin-1**

To determine the domain of SD required to interact with Nerfin-1, a series of SD deletions was made (Figure 3.3B). The ability of each of these constructs to bind to Nerfin-1 was tested *in vitro*. Constructs containing amino acids 220 – 344 of the SD protein were able to pull down Nerfin-1 (Figure 3.3C; lanes 3, 4 and 9). All these constructs contain the VID, which is the same domain utilized by SD to interact with the VG protein. Interestingly, partial deletion of the C-terminal half of the VID severely reduces the ability of the SD protein to interact with Nerfin-1 (Figure 3.3C; lane 6), while removing the N-terminal half of the VID completely eliminated the ability of the SD protein to pull down Nerfin-1 (Figure 3.3C; lane5). SD proteins lacking the VID or amino acids 220 to 344 were unable to co-immunoprecipitate Nerfin-1 (Figure 3.3C; lanes 5, 7 &8).

**Identifying the Region Nerfin-1 Utilizes to Interact with SD**

To determine the region of Nerfin-1 needed to facilitate the interaction with SD, a series of Nerfin-1 5' deletions was created (Figure 3.4A). Each of these deletion constructs was fused to a C-Terminal 6X HIS tag and transfected into S2 cells along with the N-terminal FLAG tagged full length SD gene. The S2 cells were lysed, incubated on 5% Ni NTA Magnetic Beads, washed, run on a SDS/PAGE gel and analyzed on a Western blot. The presence of the SD protein was visualized with a FLAG antibody. The use of the FLAG antibody to visualize SD reveals two species of SD, a phosphorylated and an

unphosphorylated form (unpublished data). Removing any of the first 182 amino terminal amino acids had no effect the on the ability of Nerfin-1 to co-immunoprecipitate SD (Figure 3.4B; lanes 2 - 4). Removing amino acids 182 – 310 compromised the ability of the Nerfin-1 protein to interact with SD (Figure 3.4B; lanes 5 and 6). Thus, the region between amino acids 182 – 310 is required for Nerfin-1 to bind to SD. Alignment of this region using other EIN homologs reveals two conversed regions: A novel domain composed mainly of highly charged amino acids and an EIN zinc finger domain (Figure 3.5A). To determine if the novel domain is required for the Nerfin-1/SD interaction we deleted eight amino acids from this motif. Deletion of these eight amino acids completely abolished the ability of Nerfin-1 to co-immunoprecipitate the SD protein (Figure 3.5B).

**SD alters the Function of Nerfin-1**

In the embryo, the Nerfin-1 protein is required for the longitudinal connective neurons to project across the segmental boundaries (Figure 3.6A). The longitudinal connective neurons in Nerfin-1 null embryos accumulate and tangle at or near the segmental boundary (Kuzin et al., 2005; Lukowski et al., 2006). To determine if the SD protein can alter the function of Nerfin-1, we ectopically expressed the SD protein using a Nerfin-1 Gal4 driver. Ectopic expression of the SD protein did not alter the identity of the neurons but did prevent the longitudinal connective neurons from projecting across the segmental boundaries (Figure 3.6B). The phenotype caused by over-expressing SD is similar to that seen in Nerfin-1 null embryos (Kuzin et al., 2005; Lukowski et al., 2006), indicating that ectopically expressed SD protein can interfere with the ability of the Nerfin-1 protein to properly function in the embryo.

DISCUSSION

TEAD proteins are generally widely expressed (Campbell et al., 1992; Stewart et al., 1996), and their function is dependent on the cofactors that they interact with (Belandia and Parker, 2000; Butler and Ordahl, 1999; Chen et al., 2004; Gupta et al., 2001; Gupta et al., 1997; Halder et al., 1998; Maeda et al., 2002; Simmonds et al., 1998; Vassilev et al., 2001). These cofactors are able to influence the target genes that the TEAD complexes activate. In mammals, several different cofactors are known to interact with the TEAD proteins. Of these, only two (VG and Yorkie) have been tested against the Drosophila TEAD protein. Both of them have been shown to interact with SD biochemically and genetically (Bandura and Edgar, 2008; Halder et al., 1998; Simmonds et al., 1998; Wu et al., 2008; Zhang et al., 2008). Herein, a new class of cofactors that can associate with TEAD proteins, Nerfin-1, has been identified.

To date, TEAD cofactors have been shown to interact with three different regions of the TEAD protein. The first region is located within the TEA/ATTS DNA binding domain (Gupta et al., 2001). Another region is located in the C-terminal half of the protein and is referred to as the vestigial interaction domain (VID). The majority of the cofactors have been shown to associate with this region of the protein (Chen et al., 2004; Maeda et al., 2002; Vassilev et al., 2001; Vaudin et al., 1999). The third region encompasses the entire

C-terminal end of the protein and includes the VID (Vassilev et al., 2001). Our data show that the Nerfin-1 protein binds to the SD protein via the VID.

Despite the fact that several different cofactors have been shown to interact with TEAD proteins, very little is known about the sequence required to recognize and bind to the VID. Both the VG and VG-like proteins utilize the SID to interact with the VID (Chen et al., 2004; Vaudin et al., 1999). A previous alignment of this region, revealed a consensus sequence of VDEHFRALG that is able to interact with SD (Chen et al., 2004). We have been able to localize the region required for Nerfin-1 interaction with SD to a 120 amino acid sequence located in the middle of the Nerfin-1 protein. Using a protein alignment with all the known EIN homologs, two conserved regions are seen within this 120 amino acid sequence (Figure 3.5A). Deletion of the N-terminal conserved sequence prevented Nerfin-1 from binding to with SD (Figure 3.5B), indicating that this region is critical for these two proteins to interact with each other. Comparing the conserved sequence of this region ($RKL^{H}/_{N}FEDEV$) with that from the VG and VG-like proteins reveals that six to seven of the nine amino acids are conserved, However, the order of the sequence is not conserved. Interestingly, if the sequence is inverted and then an alignment is performed, the integrity of the order is much stronger (Table 3.1). It is unclear whether this result is a coincidence or actually represents an inverted SID. If the sequence is indeed inverted, it would indicate that a particular amino acid sequence is required to interact with the VID. However, if the inversion is not functionally important, then our results would suggest that proper binding to the VID is more sensitive to the overall biochemical properties of the motif rather then amino acid sequence, or that only a few key critical amino acids are required for the domain to interact with the VID.

The cofactor Yorkie interacts with the entire C-terminal end of TEAD proteins (Vassilev et al., 2001). This region includes the entire VID. An alignment of the Nerfin-1 and Yorkie scalloped interacting domains, reveals that these domains are partially conserved (Table 3.1). Interestingly, only the N-terminal half of the sequence is perfectly conserved with the Nerfin-1 interaction domain. Because the Nerfin-1 and Yorkie proteins bind to different but overlapping regions of the SD protein, one would expect the SID of these two proteins to be only partially conserved.

The interaction with different cofactors is thought to be important in directing the TEAD proteins to different enhancers. For example, the association with the VG cofactor is thought to cause a conformational change in the SD protein that causes the protein to preferentially bind to wing specific enhancers (Halder and Carroll, 2001). The variation between the SID domains seen in Nerfin-1 and VG is highly conserved within different species (Chen et al., 2004), and may be required to elicit different conformation changes to the SD protein. These alternate conformational changes may be important for directing the TEAD proteins to different binding sites.

Nerfin-1 was originally described as a transcription factor that is important for embryonic neuronal development (Stivers et al., 2000). Mutations to the Nerfin-1 gene in *Drosophila melanogaster* alter the migratory path of neurons (Kuzin et al., 2005). The Nerfin-1 gene is also expressed in non-neural tissues, but its functions in these tissues are unknown (Stivers et al., 2000). The co-expression of the *C. elegans* Nerfin-1 and SD

homologs can alter the identity of neural cells (Wu et al., 2001). Thus, in addition to facilitating proper neural migration, Nerfin-1 may also play a role in neural differentiation. Previous work done with SD and VG reveals that these two proteins together form a selector complex (Halder et al., 1998). The ectopic expression of VG in the eye disc is able to transform the identity of these cells from an eye fate to a wing fate (Kim et al., 1997). This cell fate transformation is not seen when SD and Nerfin-1 are ectopically expressed. Rather, the ectopic expression of SD in Nerfin-1 embryonic nerve cells hinders the ability of Nerfin-1 to properly function. The same is true when Nerfin-1 is ectopically expressed in the wing pouch. Thus, unlike VG, the SD/Nerfin-1 complex does not seem to specify cell fate. The exact role of this complex is still unclear. SD and Nerfin-1 are both expressed in lamina precursor cells, and posterior to the morphogenic furrow in the eye imaginal disc. Antagonizing the SD protein with a dominant negative transgene has previously shown that SD is critically required for the proper development of these tissues (Garg et al., 2007). Thus, it is possible the SD/Nerfin-1 complex helps facilitate the proper development of either or both of these tissues.

Table 3.1. Conservation between the Scalloped interacting domains of Nerfin-1, VG and Yorkie

| | |
|---|---|
| A. EIN and VG SID | R K L FEDEV- |
| | V $^{E}/_{D}$ $^{E}/_{D}$ HFRALG |
| B. EIN and Inverted VG SID | RKLFE- **D E V** |
| | GLARFH $^{E}/_{D}$ $^{E}/_{D}$ V |
| C. EIN and Yorkie SID | **RKL**FEDEV |
| | **RKL**PNSFF |

The minimal SID domain of the VG, EIN and Yorkie family of cofactors are shown. A. There is no sequence conservation between the minimal SID from the VG and EIN family of proteins. B. An alignment when one of the domains is inverted shows a higher level of sequence conservation. C. Only the N-terminal 3 amino acids are conserved when the EIN consensus SID is aligned with the Yorkie SID. Conserved amino acids are in bold.

FIGURE 3.1. The expression pattern of *sd* and Nerfin-1in third instar larvae.

A-D) Lac Z staining of an enhancer trap allele of *sd*. A) The *sd* gene is transcribed in the distal tip of the leg imaginal disc. B) In the wing imaginal disc, *sd* is present in the pouch and along the D/V margin. C) In the eye imaginal disc *sd* is expressed posterior to the morphogenic furrow. D) *sd* is transcribed throughout the optic lobe, including the lamina precursor cells (arrowhead). E-F) Nerfin-1 expression. E) Cells posterior to the morphogenic furrow express Nerfin-1. F) Nerfin-1 is present through out the optic lobe, including the lamina precursor cells (arrow head).

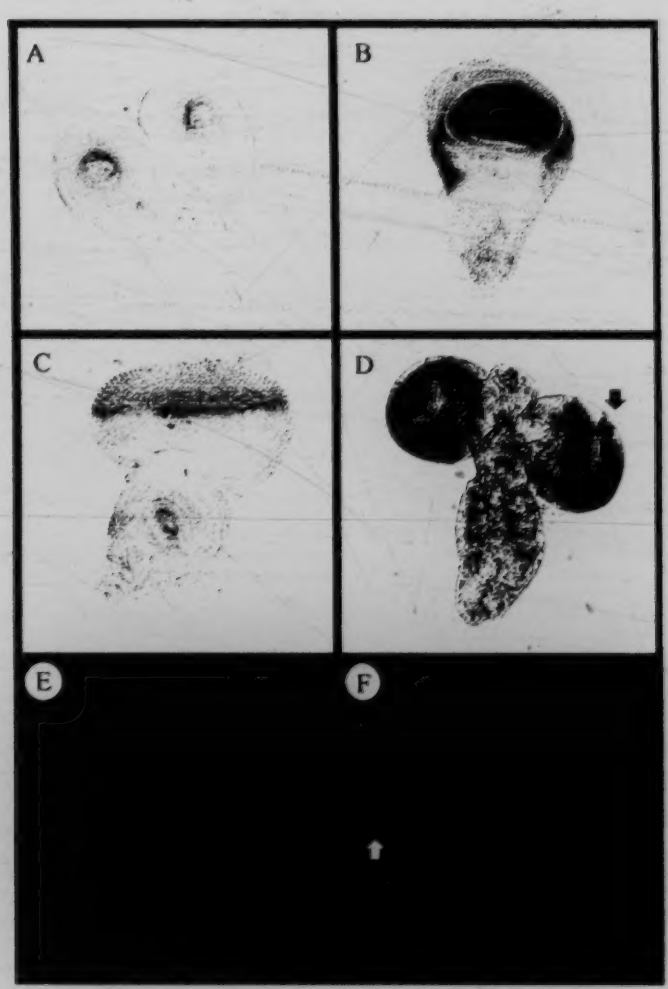

A
B
C
D
E
F

FIGURE 3.2. Over-expression studies demonstrate that Nerfin-1 interacts with SD *in vivo*.

A) Wild type adult wing. B) Using a *vg* Gal4 driver, the VGΔACT transgene is expressed along the dorsal ventral axis of the wing imaginal disc. The ectopic expression causes a loss of wing bristles and wing tissue (B compared to A). C) Co-expression of the VGΔACT with *UAS sd* rescues the dominant negative phenotype. D) Ectopic expression of Nerfin-1 in the wing imaginal disc causes a more extreme but similar loss of wing tissue as that seen with the VGΔACT transgene. E) Similar to VGΔACT, the ectopic expression of Nerfin-1 can be rescued by co-expressing *UAS sd*. F) Excess SD alone causes a loss of wing tissue as well.

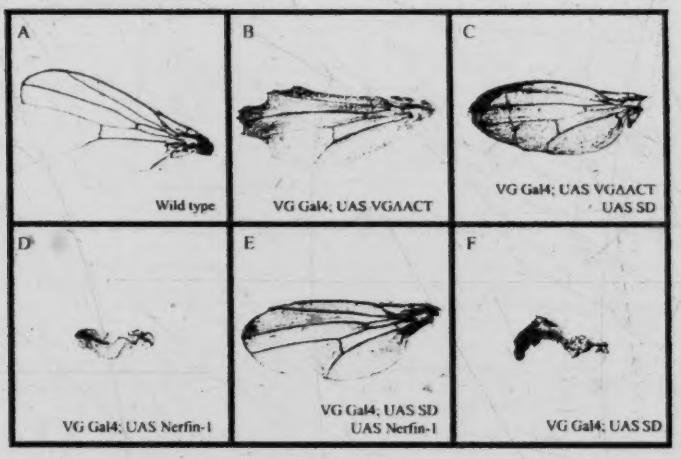

A
Wild type
B
VG Gal4; UAS VGAACT
C
VG Gal4; UAS VGAACT
UAS SD
D
VG Gal4; UAS Nerfin-1
E
VG Gal4; UAS SD
UAS Nerfin-1
F
VG Gal4; UAS SD

FIGURE 3.3. Co-immunoprecipitation experiments identify the Nerfin-1 binding domain in SD.

A) The interaction between SD and Nerfin-1 was confirmed by an *in vitro* Co-IP experiment. S2 cells were transfected with Nerfin-1, FLAG SD, Nerfin-1 and FLAG SD, or Nerfin-1 and untagged SD expression vectors. The cell extracts and the agarose beads conjugated to FLAG antibodies were incubated overnight at 4 $^0$C. The beads were washed and heat denatured and run on an 8% acrylamide gel. The presence of Nerfin-1 protein was identified by Western blot analysis using a polyclonal Nerfin-1 antibody. B) The diagram shows the set of SD deletion constructs used in C). Grey boxes highlight the two conserved functional domains: the TEA/ATTS DNA binding domain (aa 88 – 159) and the Vestigial interaction domain (VID)(aa 220 – 344). Each of the SD constructs contains a 5' FLAG tag. The first 159 and 310 amino acids are missing in the SDΔ159 and SDΔ310 constructs, respectively. The C-terminal 177, 220 and 280 amino acids were removed in the SDΔ177C, SDΔ220C and SDΔ280C reagents. Only the amino acids from position 220 to 344 are retained in the SD 220-344 construct. C) Nerfin-1 binds to the VID of SD. S2 cells were co-transfected with each of the SD deletion expression vectors and Nerfin-1 plasmid. Cells extracts were incubated with agarose beads conjugated to FLAG antibodies overnight at 4 $^0$C, washed, heat denatured and run on an 8% acrylamide gel. The presence of Nerfin-1 was confirmed by a Western blot using the Nerfin-1 antibody. Lysates from cells in Lanes 2 – 8 were co-transfected with Nerfin-1 and a FLAG tagged SD construct, while the cells from lane one were only transfected with a single construct – FLAG tagged SD Full. Constructs retaining the VID (lanes 3,4 and 9) were able to interact with Nerfin-1. Very weak binding to Nerfin-1 was seen when the C-terminal half of the VID was deleted (lane 6). Constructs missing part of or the entire VID failed to interact with Nerfin-1 (lanes 5, 7 & 8).

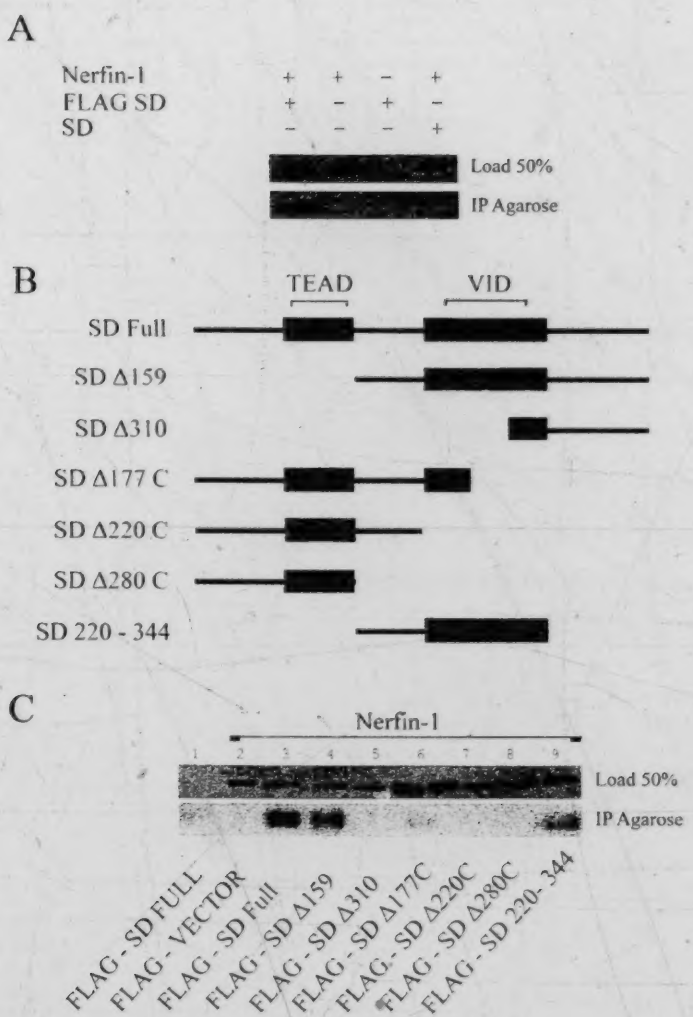
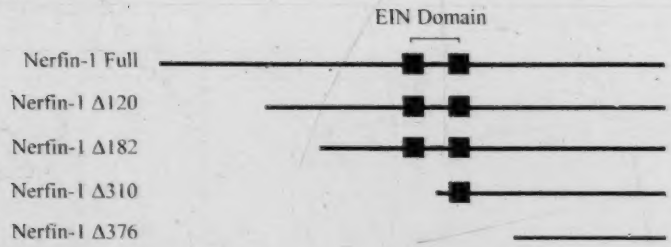

A
Nerfin-1
FLAG SD
SD
+ + − +
+ − + −
− − − +
Load 50%
IP Agarose
B
TEAD
VID
SD Full
SD Δ159
SD Δ310
SD Δ177 C
SD Δ220 C
SD Δ280 C
SD 220 - 344
C
Nerfin-1
1 2 3 4 5 6 7 8 9
Load 50%
IP Agarose
FLAG - SD FULL
FLAG - VECTOR
FLAG - SD Full
FLAG - SD Δ159
FLAG - SD Δ310
FLAG - SD Δ177C
FLAG - SD Δ220C
FLAG - SD Δ280C
FLAG - SD 220- 344

FIGURE 3.4. Co-immunoprecipitation experiments reveal the SD interacting domain of Nerfin-1.

A) The diagram shows the set of Nerfin-1 deletion constructs used in B. Each of the Nerfin-1 constructs contained a 3' 6XHIS tag. The two zinc finger EIN domains (aa 278 – 300 and aa 334 – 356) are indicated by the grey boxes. Each of the constructs contain a 5' deletion. B) S2 cells were transfected with each of the HIS tagged Nerfin-1 deletion constructs and FLAG tagged SD. The cell extract and 5% Ni NTA Magnetic Beads were incubated overnight at 4 $^{0}$C. The beads were washed, heat denatured and run on a standard 8% acrylamide gel. The presence of the SD protein was confirmed by Western blot analysis using a FLAG antibody. Lysates from cells in Lanes 2 – 6 were co-transfected with the FLAG tagged SD Full and a 6X HIS tagged Nerfin-1 construct, while the cells for lane one were only transfected with a single construct – 6X HIS tagged Nerfin-1 full. Constructs retaining amino acids 182 – 310 (lane 2 – 4) were able to Co-IP SD. Constructs missing this region failed to pull CoIP SD (lanes 5-6)

A


EIN Domain
Nerfin-1 Full
Nerfin-1 Δ120
Nerfin-1 Δ182
Nerfin-1 Δ310
Nerfin-1 Δ376

B

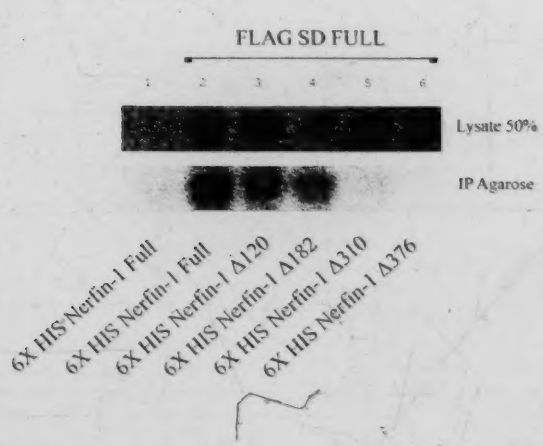
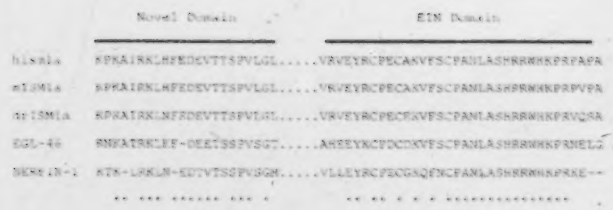

FLAG SD FULL
1
2
3
4
5
6
Lysate 50%
IP Agarose
6X HIS Nerfin-1 Full
6X HIS Nerfin-1 Full
6X HIS Nerfin-1 Δ120
6X HIS Nerfin-1 Δ182
6X HIS Nerfin-1 Δ310
6X HIS Nerfin-1 Δ376

Figure 3.5. Localizing the Nerfin-1 SD interaction domain

A) Using the amino acid sequence of Nerfin-1 and its homologs, an alignment of the region required to interact with SD was performed. The alignment shows that two conserved motifs are found within this region. The conserved amino acids are marked by an asterisk. One of the conserved domains is the EIN domain (a.a. 278 – 300). The second domain (a.a. 182 – 205) does not belong to any previously identified family. B) S2 cells were transfected with FLAG tagged SD and either full length Nerfin-1 or Nerfin-1 cDNA containing a 24 base pair deletion (NerfinΔND). The deleted amino acids are R•K•L•N•E•D•T•V. The cell extracts and the agarose beads conjugated to FLAG antibodies were incubated overnight at 4 $^{0}$C. The beads were washed and heat denatured and run on an 8% acrylamide gel. The presence of Nerfin-1 protein was identified by Western blot analysis using a polyclonal Nerfin-1 antibody. Deleting the eight amino acids prevented the SD protein from co-immunoprecipitating Nerfin-1 (lane 2)

A


Novel Domain
EIN Domain

B

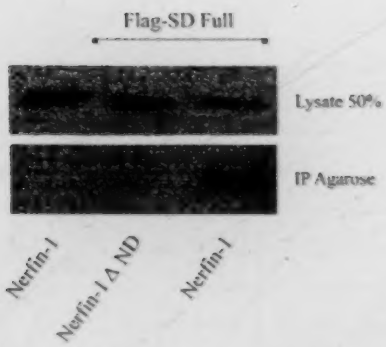
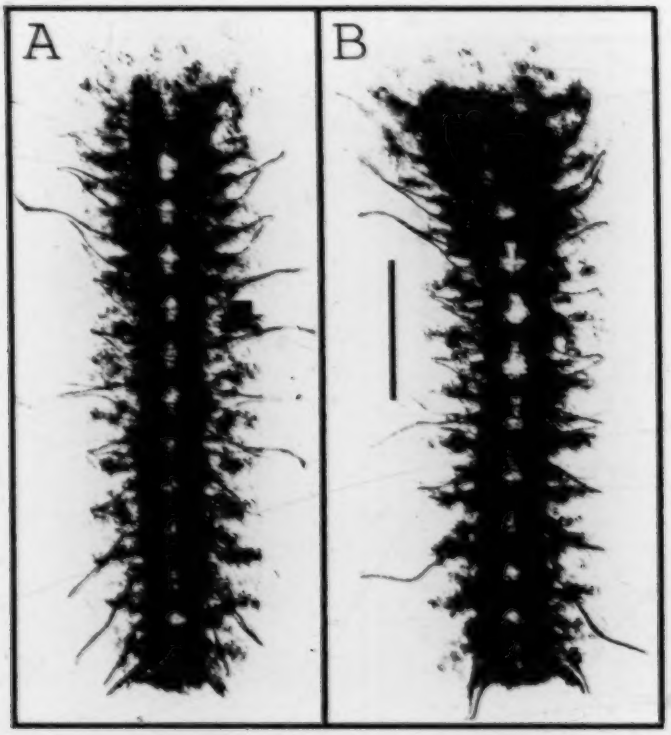

Flag-SD Full
Lysate 50%
IP Agarose
Nerfin-1
Nerfin-1 Δ ND
Nerfin-1

Figure 3.6. The SD protein is able to antagonize Nerfin-1 function

The axons of stage 15 embryos were stained with anti-Fas2 antibody. A) The ventral cords of a wild-type embryo are visible. The longitudinal connective neurons project across the segmental boundaries (arrow). B) Expressing full length SD protein with a Nerfin-1 Gal4 driver prevents some of the longitudinal connective neurons from projecting across the segmental boundaries (segments marked by a solid line).


A
B

REFERENCES: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## CHAPTER IV – Re-examining the DNA target selectivity of Scalloped

### INTRODUCTION

During development, a class of genes, termed field selector genes, often directs the fate of cells. For example, in *Drosophila*, the *eyeless* (*eye*) gene instructs cells to adopt an eye fate (Halder et al., 1995; Quiring et al., 1994), while *scalloped* (*sd*) and *Distal-less* (*dll*) promote wing (Halder et al., 1998; Kim et al., 1996; Simmonds et al., 1998; Williams et al., 1991) and limb identity (Gorfinkiel et al., 1997), respectively. While the expression patterns of some of these field selector genes are restricted, many of them are expressed in several different tissues and have multiple roles throughout the course of development. For example, the Eyeless protein is also required for the proper development of the adult central nervous system (Callaerts et al., 2001), while the Scalloped protein is also essential for promoting cell proliferation (Bandura and Edgar, 2008; Wu et al., 2008; Zhang et al., 2008).

In order to direct different developmental processes, field selector genes must activate different subsets of genes in a tissue specific manner. The proper development of the wing requires SD to activate transcription of the *wingless* (*wg*) (Liu et al., 2000; MacKay et al., 2003), *cut* (*ct*) (Halder et al., 1998; Liu et al., 2000) and *vestigial* (vg) genes (Kim et al., 1996), while promotion of cell proliferation is dependent on SD inducing expression of the *inhibitor of apoptosis-1* gene (d*IAP-1*) (Wu et al., 2008; Zhang et al., 2008). Association with various cofactors is a method by which field selector proteins activate different subsets of genes. For example, when VG binds to SD, the complex promotes expression of the wing specific subset of genes (Halder and Carroll, 2001). In contrast, when the cofactor Yorkie interacts with SD, dIAP1 expression is induced (Zhang et al., 2008).

Cofactors are able to alter the genes that selector proteins activate in several different ways. A common method is to alter the DNA sequences that the selector protein's DNA binding domain recognizes. This can be accomplished in a few different ways. One method is for the cofactor to alter the affinity a selector protein has for a particular DNA sequence. For example, the association of the selector protein Labial (Lab) to its cofactor Extradenticle (Exd) causes a conformational change in the Lab protein so that the hexapeptide, an inhibitory domain, is no longer in contact with the Lab DNA binding homeodomain (Chan et al., 1996). The disassociation of the hexapeptide from the homeodomain increases the affinity with which the Lab protein binds to DNA. A second method involves the cofactor altering the consensus sequence that the selector protein prefers to recognize. Several different studies have shown that Hox proteins as monomer recognizes a particular set of sequences, but a Hox/Exd heterodimer recognizes a different set of sequences (Chan and Mann, 1996; Chan et al., 1996; Chang et al., 1996; Phelan and Featherstone, 1997; Shen et al., 1996; Wilson and Desplan, 1999). Finally, cofactors themselves can also bind to DNA. Thus, depending on the sequence the cofactors themselves bind to, this can alter the sequences that the cofactor/selector protein complex associates with (Knoepfler et al., 1996; Ryoo and Mann, 1999).

In the wing, the SD protein is known to bind to two different cofactors, VG (Halder et al., 1998; Simmonds et al., 1998) and Yorkie (Wu et al., 2008; Zhang et al., 2008). The VG and Yorkie cofactors are unable to bind to DNA on their own, but do contain activation domains (MacKay et al., 2003; Vaudin et al., 1999; Yagi et al., 1999). The SD protein is thought to bind to a different enhancer depending on the cofactor it associates with. *In vitro* data show that the SD protein on its own is able to bind to the consensus binding site of $^{T}/_{A}{}^{A}/_{G}{}^{A}/_{G}{}^{T}/_{A}$ AT $^{G}/_{T}$ (Halder and Carroll, 2001; Jacquemin et al., 1997). Furthermore, when the SD protein is complexed to the VG or Yorkie cofactors, it is still able to recognize and bind to the consensus sequence. Interestingly, when bound by the VG protein, the SD complex only binds to sequences found in wing specific enhancers (Halder and Carroll, 2001). The complex is no longer able to associate with non-wing specific enhancer elements, even though both sequences contain a consensus binding site. This suggests two things. The first is that the VG cofactor is necessary and sufficient to discriminate between wing and non-wing enhancer sets. The second is that there must be some information in the wing enhancers that allows the SD/VG complex to distinguish it from other enhancers. This distinguishing information could be coded in the actual sequence; perhaps a particular variation of the consensus sequence is only found in wing enhancers. Alternatively, it could be in the architectural layout of the binding site. For example, all the identified SD/VG wing enhancer elements have two tandem binding sites separated by a single nucleotide (Guss et al., 2001; Halder and Carroll, 2001).

The previous work done *in vitro* leads to three important conclusions. The first is that the SD/VG complex binds with a higher affinity to wing enhancers than the SD protein alone. The second is that the two terminal domains of the VG protein are not required to bind to the SD protein in solution, but are necessary for the SD/VG complex to interact with wing enhancers. Deletion of both of these domains results in a complete loss in the ability of the complex to associate with any consensus binding site. The third and final finding is that the SD/VG complex forms as a tetramer on the wing enhancers (Halder and Carroll, 2001).

Herein, data are presented that agree with the above *in vitro* findings *in vivo*. The data are consistent with the notion that the VG cofactor does not alter the sequence that the SD protein recognizes, but rather alters the selector protein's affinity for DNA. Furthermore, it is likely that the architecture of the enhancer is what allows the SD/VG tetramer to distinguish wing enhancers from non-wing enhancers. Finally, the data provide evidence indicating that in addition to promoting the formation of the tetramer and activating transcription, the two terminal domains of VG have yet to be identified functions.

## MATERIALS AND METHODS

### Fly Stocks

Flies were raised on standard media at room temperature. *Yellow white* flies were used as controls. The $sd^{58d}$ stock harbour a hypomorphic allele that contains a P-element insertion. The UAS *sd* and UAS *sd*Δ200 constructs are described in Chow *et al.* (2004). The *vg*ΔACT strain is outlined in Garg *et al.* (2007) and the UAS *vg* is described in Mackay *et al* (2003). The *ptc* Gal4 and *vg* GAL4 lines were a gift from S. Carroll. The

*yw* Δ2-3/*Sb* flies were a gift from A. Simmonds. The UAS p35 line was obtained from the stock center.

Construction of the 2XVGΔACT:

The VGΔACT sequence was fused to itself to make a construct with a tandem SID motif. Using a polymerase chain reaction (PCR) protocol, the nucleotides that encode amino acids 171-335 of the VG protein were amplified with and cloned into the pDONR 221 P1-P4 and pDONR 221 P4-P3r vectors using a BP Clonase enzyme II mix. The resulting pDONR 221 P1-P4 and pDONR 221 P4-P3r vectors were mixed with a STOP pDONR221 P3-P2 vector (A vector containing an in-frame stop codon). These three plasmids were recombined using the LR enhanced Clonase II mix onto the pTFW injection vector. Because the resulting vector contains a repeat, the resulting plasmids were transfected and propagated in Stble II cells. Vectors containing repeats are more stable in Stble II cells (See Appendix I for list of primers used).

Construction of the 5'VP16::VGΔN, VGΔC::3'VP16

The VP16 activation domain was amplified using PCR and cloned into both the pDONR 221 P1-P4 and pDONR 221 P3-P2 vectors using the BP Clonase enzyme II mix. The VP16 inserted into the pDONR221 P3-P2 vector contains a stop codon at the end of the sequence. The sequence encoding the first 65 amino acids of the VG protein was also amplified using PCR and cloned in the pDONR221 P1-P4 vector. The sequence encoding the last 103 amino acids of the VG protein was cloned in the pDONR221 P3-P2 vector. Using the LR enhanced Clonases II enzyme mix, the following plasmids VP16 pDONR 221 P1-P4, the VGΔACT pDONR221 P4-P3r and the pDONR221 P3-P2 vector containing the sequence encoding the last 103 amino acids of the VG protein together were recombined onto the pTFW injection vector to generate the 5'VP16::VGΔN construct. The VGΔC::3'VP16 vector was generated the same way as the 5'VP16::VGΔN construct, except the VGΔACT pDONR221 P4-P3r, VP16 pDONR 221 P3-P2 and the pDONR 221 P1-P4 vector containing the sequence coding for the first 63 amino acids of VG were used.

Construction of the 5'VP16::SD and SD::3'VP16

The SD cDNA was cloned in to the pDONR 221 P3-P2 vector. The stop codon was removed from the SD cDNA during the PCR step. Using the LR enhanced Clonases II enzyme mix, the following plasmids VP16 pDONR 221 P1-P4, the SD pDONR 221 P4-P3r and the STOP pDONR 221 P3-P2 vector were recombined onto the pTFW injection vector to generate the 5'VP16::SD construct. The generation of the SD::3'VP16 construct involved recombining an empty pDONR 221 P1-P4, the SD pDONR 221 P4-P3r and the VP16 pDONR 221 P3-P2 vectors onto the pTFW injection vector.

PCR conditions:

Invitrogen Hi Fi platinum *Taq* was used in standard PCR conditions. The template was allowed to initially denature at 94° for 5 min. DNA was amplified with 30 cycles at 94°

for 30 sec (denaturing) followed by 55° for 30 sec (annealing) and 68° for 90 sec (extension).

Micro-injections:

Micro-injections were performed as described in Rubin and Spradling (1982). Each of the pTWF vectors were injected into *yw* flies carrying an endogenous copy of the Δ2-3 gene on the third chromosome. The emerging flies were crossed to *yw* flies. The F1 generations were scored for pigmented eyes.

Immunocytochemistry.

Imaginal discs were dissected from third instar larvae and fixed for 20 min in 2.5 % paraformaldehyde and 5% DMSO in PBS. Discs were stained with rabbit anti-VG antibody (S. Carroll) at 1:100 dilution and Mouse anti-Wg antibody 4D4 (Developmental Studies Hybridoma Bank) at 1:200.

RESULTS

**Over-expressing SD suppresses downstream wing-specific enhancers**

Proper wing development is sensitive to the SD:VG ratio. Over-expression of the SD protein in the wing disc causes a dominant negative phenotype, whereas increasing the amount of the VG protein produces a slightly overgrown wing (Simmonds et al., 1998). There are at least two possible reasons why excess SD may lead to a loss of wing tissue. The first is that cells in the wing pouch are sensitive to the levels of SD. High concentrations of the SD protein may be toxic and cause cell death. Another explanation is the presence of excess SD protein may interfere with the activation of downstream genes. To determine if the latter explanation is true, the expression of the SD downstream target genes, *wg* and *vg*, was examined. Two different enhancers are known to control the expression of the *vg* gene. The SD/VG complex only binds to the quadrant enhancer, which is responsible for directing expression of the *vg* gene in each of the quadrants of the wing pouch (Figure 4.1A). The other *vg* enhancer, the boundary enhancer, does not contain any SD binding sites and directs the expression of *vg* along the dorsal/ventral (D/V) boundary (Kim et al., 1996). In the wing, the SD/VG complex is only required to maintain the expression of WG along the D/V boundary, but not along the periphery of the wing pouch (Figure 4.1B). The D/V boundary cells express both the VG and WG proteins (Figure 4.1C). The anti-apoptotic factor p35 was co-expressed with SD to ensure partial survival of the wing pouch. The resulting wing imaginal discs only had VG expression along the D/V boundary (Figure 4.1D) and WG protein along the periphery of the wing pouch (Figure 4.1E). These patterns indicate that the SD/VG dependent enhancers of the *vg* and *wg* genes are suppressed when SD is over-expressed in the wing disc (See Figure 4.1F for the overlay showing the expression patterns that indicate this suppression).

**The excess SD protein competes with the SD/VG complex for wing specific binding sites**

The transcriptional repression of the downstream target genes observed upon by over-expressing SD could be caused by the excess SD protein interfering with the ability of the endogenous SD/VG tetramer to form, or by the unbound SD protein competing with the SD/VG tetramer for the wing specific enhancer sites. To distinguish between these two possibilities, the sequence encoding the VP16 activation domain was cloned into the 5' and 3' ends of the *sd* gene. If the excess SD protein binds to and represses expression of the downstream wing genes, the addition of the VP16 activation domain should prevent the SD protein from acting as a repressor and inhibiting wing development. However, if the extra SD protein is inhibiting the formation of the SD/VG tetramer, then expression of both of these constructs should still cause a dominant negative phenotype. Over expression of either the 5'VP16::SD or the SD::3'VP16 protein had no effect on wing development (data not shown), in that a wild-type wing was still observed. To test if these fusion constructs can still bind to DNA and activate transcription, the SD VP16 fusion proteins were expressed in a $sd^{58}$ mutant background. The $sd^{58}$ mutants have small wings and no bristles along the margin (Figure 4.2A). The SD VP16 constructs were able to induce the formation of a few wing margin bristles at the hinge and had slightly more wing blade tissue (Figure 4.2B and C).

**Verifying the *in vitro* results.**

The idea that the dominant negative phenotype is caused by excess SD protein competing for the downstream binding sites provides us with an *in vivo* system to test binding properties of the SD protein. One important finding in the previous *in vitro* experiments is that the two terminal domains of the VG protein are essential for the SD/VG complex to bind to DNA. Deletion of these domains still allows the protein to bind to SD, but prevents the complex from binding to both wing specific and non-specific sites. To verify this *in vitro* result, an attempt to rescue the dominant negative phenotype (Figure 4.3B) was done by co-expressing the VGΔACT protein. The two terminal domains of VG have been removed in the VGΔACT construct. If the *in vitro* results also apply to the *in vivo* system, co-expression of the VGΔACT construct with SD should rescue the dominant-negative wing phenotype. Indeed, this is the case. The over-expression of SD with the VGΔACT gene results in an almost total rescue of the wing phenotype (Figure 4.3C).

**Comparison of the wing specific SD binding sites with non-wing specific binding site**

To gain further insight into the mechanism of how VG promotes preferences for wing specific enhancers, the sequences of wing specific and non-wing specific binding sites were compared to determine if there is any variation between these two sequence sets. A small difference was seen at the third nucleotide position. Twenty of the twenty-one non-wing specific sites contain a guanine at this position. In contrast, the wing specific enhancers contain a guanine, thymine or adenine (Table 4.1). Furthermore, all the *dIAP1* enhancer binding sites contain a thymine at nucleotide position eight, whereas only a

quarter of the non-*dIAP* sites had a thymine at this position. The fact that different nucleotides are conserved in each enhancer set suggests the possibility that each SD cofactor may direct the SD/cofactor complex towards particular variations of the consensus sequence. However, an argument against this hypothesis is that the conserved nucleotides are not restricted to one set of enhancers. For example, the conserved guanine and thymine nucleotides in the *dIAP1* enhancer are also present in one of the sal-750 binding sites.

**Formation of a tetrameric complex is essential for binding to wing specific enhancers**

All the wing enhancers that are known to recruit the SD/VG complex contain two tandem binding sites (Halder and Carroll, 2001). Previous *in vitro* studies have shown that the SD/VG complex always forms as a tetramer on the wing specific binding sites. Furthermore, the SD/VG dimer is unable to form a stable interaction with any of these enhancers (Halder and Carroll, 2001). To determine if the recruitment of two SD proteins is critical in forming a stable complex on the wing enhancer sequences, a construct was created that contains two VGΔACT proteins fused together. This construct (2XVGΔACT) would have the same properties of the VGΔACT protein, however, it would recruit two SD proteins as opposed to one. The recruitment of two SD proteins should mimic the binding properties of the SD/VG tetramer. To test if the construct is functional, we first only over-expressed the 2XVGΔACT protein. Expression of the 2XVGΔACT construct in the wing causes a dominant negative phenotype (Figure 4.3D). Co-expression of the 2XVGΔACT protein in the wing with an ectopic copy of SD failed to rescue the wing phenotype (Figure 4.3E). In contrast to the VGΔACT results, when SD is bound by the 2XVGΔACT protein the complex is still able to bind to and suppress the expression of downstream wing target genes.

**The terminal domains of VG have multiple functions**

The VG protein has been previously shown to activate transcription and promote the formation of the SD/VG tetramer (Halder and Carroll, 2001; Vaudin et al., 1999). To determine if there are other functions associated with the two activation regions of the VG protein, each of them was replaced with a general VP16 activation domain (Figure 4.4A) and then expressed in the eye. Ectopic expression of the wild type VG protein in the eye disc leads to ectopic wing tissue growing from the adult compound eye (Kim et al., 1996). Thus, if the only role of the two terminal domains is to activate transcription and promote the formation of the SD/VG tetramer, replacing either of the domains with a VP16 activation domain should also cause ectopic wing tissue outgrowth in the eye. However, expression of either the 5'VP16:VG or the VG:3'VP16 failed to cause any ectopic wing tissue outgrowth (Figure 4.4B).

## DISCUSSION

In the wing, excess SD results in a loss of wing tissue (Simmonds et al., 1998). The results herein, in combination with previous *in vitro* experiments, suggest that the wing phenotype is due to the excess SD protein competing with the SD/VG complex for the same binding sites. This observation is supported by the fact that in the previously published *in vitro* experiments (Halder and Carroll, 2001), the SD protein is able to recognize both wing specific and wing non-specific binding sites. Furthermore, when the *sd* gene is over-expressed in the wing disc, only the enhancers that are dependent on the SD/VG complex are suppressed. The expression patterns from the other wing enhancers that are not driven by the SD/VG complex are unaffected (Figure 4.1). Finally, the data indicate that by adding an activation domain to the SD protein the dominant negative over-expression phenotype is lost. Although it is predicted that the dominant negative wing phenotype is lost because the SD protein can no longer act as a repressor, it is also possible that the addition of the VP16 activation domain may alter the binding properties of the SD protein. Attempts to rescue a *sd* hypomorphic allele with the SD VP16 fusion construct resulted in only a very minor rescue (Figure 4.2B and C). However, because the construct can cause minimal rescue of the wing, this suggests that the protein can, at the very least, partially bind to wing enhancers. Even if the fusion of the VP16 construct reduces the ability of the protein to bind to wing enhancers, this minimal binding should still be enough to cause a slight loss of wing tissue as certain transgenic *sd* lines display a wing notching phenotype in the absence of an UAS driver (data not shown).

The fact that the SD protein is able to bind to wing enhancers provides us with a system to test the *in vitro* observations *in vivo*. *In vitro* experiments have shown that the association of VG with SD severely reduces the ability of the dimer to bind to non-wing specific sites. It is unclear whether this decrease is due to a change in the preferred sequence that the SD DNA binding domain recognizes or a decrease in affinity for DNA. Because the association of the VGΔACT protein with SD does not allow the complex to activate transcription, and the fact that we see a rescue of the dominant negative wing phenotype when we co-express the VGΔACT and SD proteins indicates that the association with VG is altering the affinity of the complex for DNA and not the sequence that the complex recognizes.

If binding with VG decreases the ability of the SD protein to associate with DNA, then how does the protein bind to wing specific enhancers? One striking feature is that all wing specific enhancers contain two tandem binding sites (Halder and Carroll, 2001). Furthermore, *in vitro* experiments show that the SD protein cooperatively binds to DNA when only a single nucleotide separates two consensus sites. This cooperative binding is lost when the space between the two sites is greater then one nucleotide (Halder and Carroll, 2001). Thus, a complex containing two SD proteins will have a much stronger affinity for DNA then one containing a single SD protein. The observation that the 2XVGΔACT protein, a complex that is able to recruit two SD units, is not able to rescue the dominant negative phenotype, whereas the VGΔACT construct can rescue the wing, indicates that the decrease in DNA binding affinity caused by the association with the VG protein can be overcome by the cooperative binding properties of the SD protein.

These observations allow one to tweak the previous model of how VG promotes the SD protein to preferentially bind to wing specific enhancers. It can be proposed that VG promotes binding selectivity in two different ways. The first is the VG protein decreases the affinity the SD protein has for DNA. Both the *in vitro* and *in vivo* results support this idea. This idea is further supported by the fact that an alignment of the binding sites did not reveal any differences between wing specific and wing non-specific binding sites. Because of the lack of divergence between the two enhancer sets, the decrease in affinity would ensure that the SD/VG dimer does not bind to and activate the transcription of non-wing genes, since non-wing genes would only contain a single binding site. The second is that VG promotes the formation of a tetrameric complex. The importance of the tetramer is that it contains two SD proteins, which allows for cooperative binding to occur. The cooperative binding attribute would increase the affinity that the tetramer has for DNA, thus allowing it to recognize enhancers with two tandem SD/VG sites. This feature is critical, because in the model (Figure 4.5) the major distinguishing feature between the enhancer types is that wing enhancers contain two tandem binding sites. The importance of having two SD proteins is supported by the fact that the 2XVGΔACT protein does not cause the SD protein to dissociate from the enhancer, and by the *in vitro* observation that deletion of the VG domain that promotes formation of the tetramer results in a loss of the complex from recognizing wing specific enhancers(Halder and Carroll, 2001).

As previously mentioned, at least one of the terminal activation domains of VG is required for activating transcription and promoting the formation of the tetramer. Interestingly, replacing one of these regions with a general VP16 activation domain prevents the protein from functioning, as the construct is no longer able to induce ectopic wing growth in the eye imaginal disc. This result suggests that in addition to activating transcription and promoting the formation of the tetramer, the terminal domains have other roles that are critical for wing development. One possible role may to be recruit as yet unknown other cofactors to the enhancer. A previous study showed that having just the tandem binding sites is not sufficient to drive the expression of a reporter gene. The activation of a reporter gene required that the SD sites be adjacent to another transcription factor binding site such as Mad/Medea or Suppressor of Hairless [26]. Thus it is possible that VG may help recruit these proteins to enhancers and that this recruitment is facilitated by the terminal activation domains.

TABLE 4.1. Various sequence elements bound by SD

Wing specific binding sites

| | |
|---|---|
| cut-564 | TGTAATTC |
| | AAAAATGT |
| cut-341 | TAAAATTA |
| | AGAAATTA |
| sal-750 | TGGAATCC |
| | ACGAATGT |
| kni-268 | TTACATTT |
| | TCGCATAG |
| | |
| CONCENSUS | NNNNATNN |

Non-wing binding sites

| | |
|---|---|
| sal-862 | ACTTATTA |
| cTNT | AGGAATGC |
| MHC | TGGAATGA |
| 1XGT | TGGAATGT |
| | |
| *dIAP-1* | CTGAATGT |
| *dIAP-1* | TGGAATTT |
| *dIAP-1* | TTGTATCT |
| *dIAP-1* | CTGCATTT |
| *dIAP-1* | CAGTATTT |
| *dIAP-1* | GGGTATAT |
| *dIAP-1* | CTGTATTT |
| *dIAP-1* | AAGTATGT |
| *dIAP-1* | AAGTATGT |
| *dIAP-1* | AGGAATTT |
| *dIAP-1* | CGGAATTT |
| *dIAP-1* | GAGTATGT |
| *dIAP-1* | ATGTATGT |
| *dIAP-1* | TGGTATAT |
| *dIAP-1* | TAGCATTT |
| *dIAP-1* | GTGTATAT |
| *dIAP-1* | CTGTATGT |
| | |
| CONCENSUS | NNGNATNN |

All the known SD binding sites are listed. Each of the wing specific enhancers contains two binding sites separated by a single nucleotide. The non-wing specific sites are divided into two groups. The first four sites were originally isolated from mammalian systems, but are still recognized by the SD protein. The second set are all the predicted binding sites found in the *dIAP1* enhancer. The conserved nucleotides are highlighted in bold. Sequences are from Guss et al. (2001) (cut, sal, kni), Cooper and Ordahl (1985) (cTNT), Molkentin and Markham (1994) (αMHC), and Davidson et al. (1998) (1XGT).

FIGURE 4.1. Over-expression of SD leads to suppression of downstream wing target genes.

Rabbit α VG antibody and mouse α WG antibodies shows the wild type expression profile of the A) VG (green) and B) WG (red) proteins. C) An overlay of WG and VG expression. Cells in the D/V boundary express both VG and WG (yellow). The quadrant enhancer is active in the remainder of the pouch (green). D) Over expression of SD and P35 results in an absence of VG protein in the wing pouch. The VG protein is only seen along the D/V boundary. E) Part of the Wingless expression profile is also lost in the VG GAL4; UAS SD; UAS P35 flies as WG protein is no longer seen along the D/V boundary. F) An overlay of D) and E). None of the D/V cells express both WG and VG.

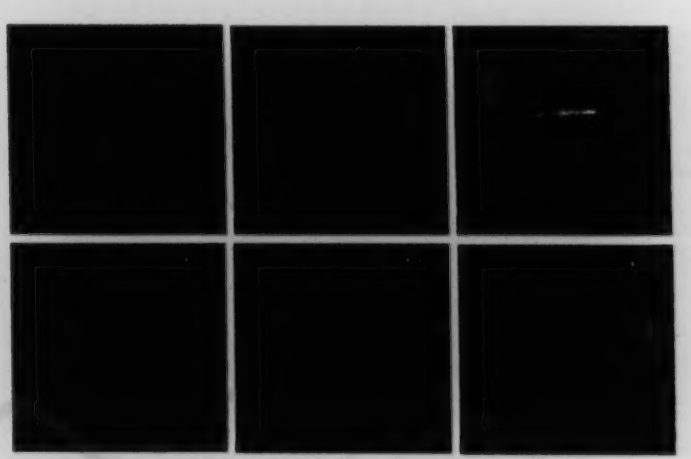


FIGURE 4.2. SD VP16 fusion protein can only partially rescue the $sd^{68}$ mutant wing phenotype

A) $sd^{68}$ mutants have very little wing tissue. B) Expression of the 5'VP16::SD fusion protein in the wing disc results in a slightly larger wing blade. Furthermore, these wings also have some bristles along the margin (arrowhead). C) A similar phenotype is seen when the SD::3'VP16 fusion protein is used to rescue the $sd^{68}$ mutant wings.

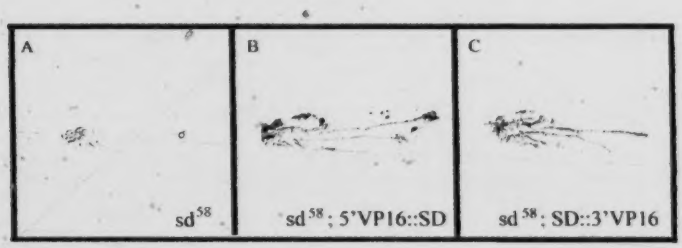

A
B
C
sd58
sd58; 5'VP16::SD
sd58; SD::3'VP16

FIGURE 4.3. Examining the *in vivo* binding properties of SD

A) Wild-type wing. B) Over expression of SD causes a dominant negative phenotype in the wing disc. C) Co-expression of the SD and VGΔACT genes leads to a dramatic rescue of the wing phenotype. D) Expressing the 2XVGΔACT with a VG GAL4 driver also causes a dominant negative phenotype in the wing. E) The co-expression of the 2XVGΔACT and SD still results in an equivalent loss of wing tissue.

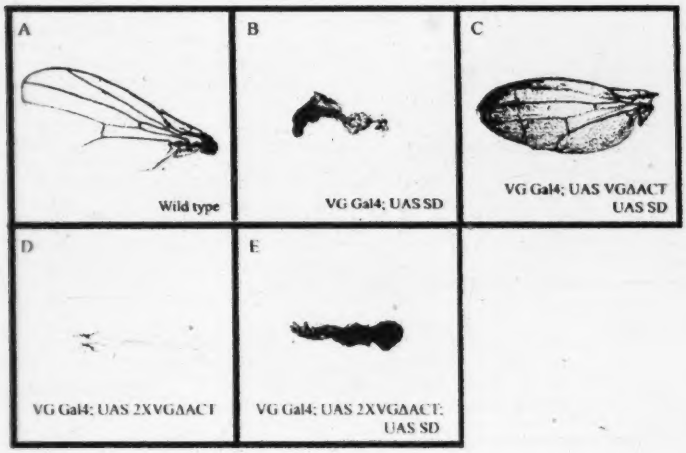
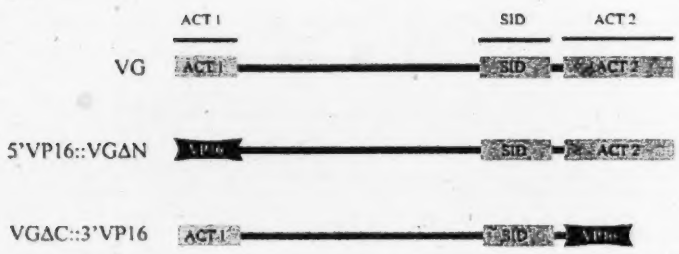

A
Wild type
B
VG Gal4; UAS SD
C
VG Gal4; UAS VGΔACT
UAS SD
D
VG Gal4; UAS 2XVGΔACT
E
VG Gal4; UAS 2XVGΔACT;
UAS SD

FIGURE 4.4. THE VP16 activation domain cannot substitute for the VG terminal activation domains

A) A schematic of the VG protein is shown. The two terminal domains, ACT1 and ACT2, are required to activate transcription and promote the formation of the tetramer. The SID is the region of the protein that physically interacts with the SD protein. Each of the ACT domains were replaced with a VP16 activation domain. The ACT1 domain was replaced with a VP16 activation domain in the 5'VP16::VGΔN construct. The ACT2 domain was replaced with a VP16 activation domain to create the VGΔC::3'VP16 construct. B) Expression of the VG:VP16 alleles was able to partially rescue the $vg^{BG}$ phenotype. The level of rescue is indicated by plus signs. A "+"indicates partial rescue, while "+++" would indicate complete rescue. The ability of the constructs to induce ectopic wing growth in eye is marked by either a "+" or a "-". Unlike the wild type copy of VG, neither of the VP16 constructs was able to promote ectopic wing tissue growth in the adult compound eye.

A

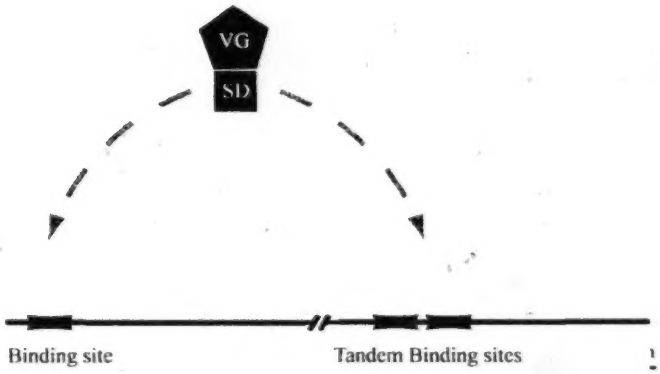


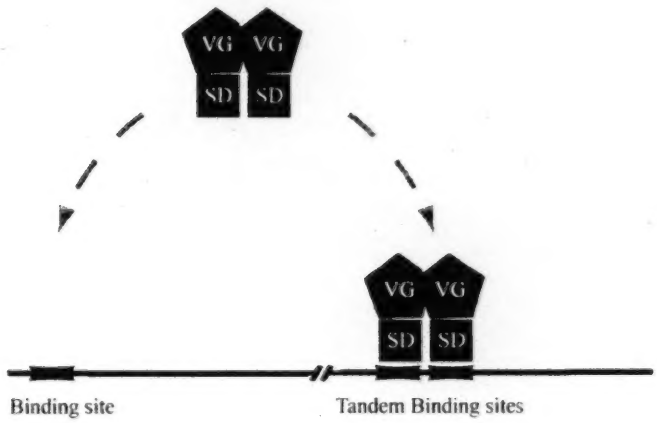

B

| Construct | Rescue $vg^{BG}$ wings | Ectopic Wing Growth |
|---|---|---|
| VG | ++ | + |
| 5'VP16::VGΔN | + | - |
| VGΔC::3'VP16 | + | - |

FIGURE 4.5. A model for SD/VG target selectivity.

A) As a dimeric complex, the SD/VG complex has very weak affinity for DNA. However, the complex is unable to recognize sites that occur on their own or as doublets. B) The presence of two DNA binding domains in the tetramer allows the complex to associate with enhancers that contain two binding sites. The tetramer is unable to associate with enhancers that contain a single binding site.


VG
SD
Binding site
Tandem Binding sites


VG
VG
SD
SD
VG
VG
SD
SD
Binding site
Tandem Binding sites

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## Chapter V - General Discussion and Future Direction

The data presented in this thesis help us to understand better how the *Drosophila* TEA/ATTS domain (TEAD) selector protein *Scalloped* (*SD*) is able to control the development of several different tissues. Enhancer trap studies show that the *sd* gene is expressed in several different tissues in the embryo and larvae (Campbell et al., 1992). Most of the past literature has focused on how the SD protein is able to regulate wing development. This thesis focuses more on the role that the SD protein plays in the other larval tissues where the *sd* gene is expressed. Furthermore, data are presented that identify a novel cofactor that is not expressed in the wing, but that can bind to the SD protein. Finally, evidence is presented that refines the model of how SD is able to regulate the expression of different sets of genes in a tissue specific manner.

Several alleles of *sd* are embryonic or pupal lethal (Srivastava and Bell, 2003). Because wings are not required for survival in laboratory cultures, the existence of these alleles suggests that SD plays a critical role in embryonic and larval development. To determine the nature of this role, a reagent (VGΔACT) was created, that is able to hinder the SD protein from interacting with other cofactors and bind to DNA. Interestingly, the expression of this reagent only affected larval development. Expression of the construct had no effect in the embryo. There are several possibilities that could explain why the reagent has no affect in the embryo. The first is that the SD protein does not interact with other cofactors to regulate the expression of embryonic genes. Because TEAD proteins need to interact with cofactors to activate transcription (Chen et al., 2004a; Chen et al., 2004b; Halder et al., 1998; Jiang et al., 2000; Maeda et al., 2002; Mahoney et al., 2005; Simmonds et al., 1998; Vaudin et al., 1999), this explanation would suggest that SD would function only as a repressor. An argument against this hypothesis is the fact that over-expressing the *sd* gene with an *actin* Gal4 driver causes embryonic lethality. However, it is unclear whether this lethality is caused by over-expressing the gene in cells that endogenously express *sd*, or if it is due to the ectopic presence of SD in cells that do not normally express this protein. The ectopic expression of SD in cells that do not normally express the protein could potentially interfere with the SD-independent roles of its cofactors. For example, data presented herein show that the ectopic expression of SD in the embryo interferes with Nerfin-1 function and causes lethality. If this is the reason for the lethality, then the SD protein could still function as a repressor in the embryo. A second possibility is that the cofactors that the SD protein interacts with in the embryo utilize a domain other then the vestigial interaction domain (VID). Although all the known SD cofactors utilize the C-terminal end of the protein (Halder et al., 1998; Simmonds et al., 1998; Wu et al., 2008), several cofactors have been shown to associate with the TEAD proteins via the DNA binding domain (Gupta et al., 2001; Gupta et al., 1997). If the embryonic cofactors are interacting via this domain, then our reagent should not have an affect on embryonic development. Another explanation could be that the cofactors that SD interacts with in the embryo are expressed at very high levels. At these levels, the VGΔACT protein is unable to compete with the endogenous cofactors and, consequently, is unable to antagonize SD function. The absence of an embryonic phenotype is an interesting result. It indicates that the SD protein functions in a different manner in the embryo and larva. This idea is further supported by the fact that mutations

in the C-terminal end of the protein, the region where the VGΔACT protein binds to SD, do affect embryonic development. In chapter two, data show that the over-expression of the SDΔ200 construct interferes with larval tissue development. One can speculate that the reagent binding to and titrating the SD cofactors causes the larval phenotypes. Thus, if one was to over express the region of SD that is required to interact with the embryonic cofactors, one could recapitulate this effect in the embryo.

In larvae, expressing the VGΔACT construct revealed that the SD protein has a critical role in the development of the wing and leg appendages, as well as the optic lobe and adult compound eye. The fact that expression of the SDΔ200 reagent causes similar phenotypes in these tissues further supports the notion that the results are due to specifically antagonizing the SD protein and not due to a secondary effect of the reagent. It would be interesting to see if a *sd* RNAi reagent (Zhang et al., 2008) could have a similar affect in the development of these tissues. The inability of the SD protein to bind to its cofactor Yorkie could partially explain some of the phenotypes caused by the VGΔACT reagent. For example, antagonizing SD function in the leg disc affects the ability of the cells to survive. This could be due to the fact that the SD/Yorkie complex is needed to induce the expression of the anti-apoptotic factor *inhibitor of apoptosis protein – 1* (d*IAP-1*) (Bandura and Edgar, 2008; Wu et al., 2008; Zhang et al., 2008). Currently, it is unclear why antagonizing SD function causes the other phenotypes. In the adult head, an ectopic cluster of cells between the lamina and the basal membrane of the ommatidia is seen when the VGΔACT reagent is under the control of an *eye* or *ptc* Gal4 driver. A key first step in understanding this phenotype would be to identify the origin of these cells. The use of specific neural and eye disc antibodies could allow one to determine if these cells originate from the optic lobe, the eye disc or both.

The *Nervous finger -1* (*Nerfin*-1) and *sd* genes are expressed in the lamina precursor cells (Campbell et al., 1992; Stivers et al., 2000). In chapter three, data are presented that Nerfin-1 and SD interact with each other. Thus, an original prediction was that the ectopic cluster of cells might result from perturbing these two proteins from interacting with each other. However, driving the SDΔ200 and VGΔACT reagents with a Nerfin-1 Gal4 driver failed to cause any phenotype. This observation does not completely discredit the possibility that these two proteins can interact. Nerfin-1 is weakly expressed in the lamina and thus it is possible that low levels of SDΔ200 and VGΔACT reagents are unable to affect the development of this tissue. The available *sd* RNAi reagent has been shown to cause a stronger dominant negative phenotype than those seen by generating mitotic clones with a lethal allele of *sd*. Thus, it would be worthwhile to obtain this *sd* RNAi reagent and drive it with the Nerfin-1 Gal4 driver.

A large-scale yeast-2-hybrid experiment predicted two novel binding partners for SD (Giot et al., 2003). The first one, Yorkie, has been shown to interact with both SD (Wu et al., 2008; Zhang et al., 2008) and the mammalian homolog, Transcription enhancer factor-1 (TEF-1) (Vassilev et al., 2001). In chapter three, data from both *in vitro* and *in vivo* experiments show that the other factor, Nerfin-1, and SD also interact with each other. Furthermore, the results show that Nerfin-1 and Vestigial (VG) use a motif of similar amino acid composition to dock to the same domain on the SD protein. An

alignment of these two motifs shows no sequence conservation. It is unclear whether there is any significance to the fact that both motifs are composed of the same amino acids. One possibility is that only the overall biochemical and physical properties of the domain are important to interact with the VID and the variation in the amino acid sequences causes different conformational changes in the SD protein. ~~One way to test~~ this hypothesis would be to replace the VG sequence with the Nerfin-1 sequence and see if the chimeric protein is still able to interact with SD and properly promote the formation of the wing. Our hypothesis would be validated if swapping of the domains still allows the VG protein to bind to SD, but prevents the chimeric protein from rescuing a *vg* mutant wing. A peptide array analysis could allow one to determine critical amino acids, and which amino acids could be substituted for one another. These data could help us better understand the sequence composition of the amino acids required for cofactors to interact with the VID. The Yorkie protein has been shown to interact with a larger portion of the C-terminal end of the SD protein than the Nerfin-1 and VG cofactors (Vassilev et al., 2001; Vaudin et al., 1999). This region includes the entire VID. Interestingly, the minimal scalloped interaction domains of Yorkie and Nerfin-1 are highly conserved at the N-terminal ends. However, there is no sequence or amino acid conservation at the C-terminal ends between these two motifs. This result is not very surprising considering that these two proteins partially recognize the same region of the SD protein.

Expression of Nerfin-1 in the wing causes a dramatic loss of wing tissue. However, unlike the hypomorphic alleles of *sd* and ectopic expression of the VGΔACT construct, the ectopic expression of Nerfin-1 fails to affect the development of bristles along the wing margin. It is unclear why Nerfin-1 is unable to inhibit the development of these bristles. One explanation for this phenotype is that the Nerfin-1/SD complex plays a role in normal bristle development. However, that seems unlikely because Nerfin-1 is not normally expressed in the wing disc, and the ectopic expression of Nerfin-1 with the *vg* GAL4 driver does not lead to the formation of these bristles outside of the margin. A second possible explanation is that Nerfin-1 associates with SD with a lower affinity than the VG-protein does. Thus, in the wing margin SD still binds to VG and promotes the formation of bristles along the wing margin. In the wing pouch, the Nerfin-1 protein is not able to prevent VG from binding to SD, but is able to out compete the cofactor Yorkie. Since the Yorkie protein is required for cell survival and growth, a dramatic loss of wing tissue is seen in the adult fly. One way to test this hypothesis would be to perform competitive co-immunoprecipitation experiments using all three cofactors.

In the eye disc, ectopic expression of the cofactor VG leads to an outgrowth of wing tissue (Kim et al., 1996). This observation indicates that the association of the VG protein to SD is able to alter cell fate. The Nerfin-1/SD complex does not seem to confer any cell fate identity. This notion is supported by the fact that ectopic expression of Nerfin-1 causes a phenotype resembling a SD null. The reciprocal is also true. Inducing *sd* expression in the CNS causes a phenotype similar to a Nerfin-1 null. Currently, it is unclear what the functional role of this complex is. As mentioned before, antagonizing this complex with our dominant negative reagents in cells that endogenously express both proteins does not seem to affect development. However, as previously indicated, the lack

of a phenotype may be due to the fact that a weak driver was used to express our reagents. In *C. elegans*, these two proteins are required to inhibit the formation of touch cells. Double mutants in these two genes lead to the development of extra touch cells (Wu et al., 2001). Thus, one possible role for the SD protein may be to inhibit Nerfin-1 function. The use of a selector protein as an inhibitor is not a novel concept. The Sloppy-paired and Engrailed proteins have previously been shown to inhibit the function of the Sex-combs reduced/Extradenticle transcription factor complex (Gebelein et al., 2004). Furthermore, although little is known about Nerfin-1 function, previous reports have shown that the Nerfin-1 transcripts are highly regulated in the embryo (Kuzin et al., 2007). The *Nerfin-1* gene is transcribed in several different neural cells, but only translated in a few of them. Thus, the inhibitory affect of SD may be another level of regulating Nerfin-1 function.

In chapter two it was shown that the larval tissues that SD helps pattern are sensitive to the levels of SD protein. Because we do not know what cofactors SD binds to in many of these tissues, one cannot be sure if these cells are sensitive to the absolute levels of SD or the ratio of the SD protein to a specific cofactor. In the wing disc, the cells are actually sensitive to the ratio of SD to VG. To determine why these cells are sensitive to this ratio, expression of downstream wing target genes was examined. Over expressing SD in the wing led to the suppression of these target genes. The data presented in chapter four suggest that the extra SD competing with the endogenous SD/VG complex for the wing specific binding sites results in the suppression of these downstream genes.

In chapter two data are also presented that demonstrate that the SD protein is required for the development of several different tissues. The proper development of these tissues requires the SD transcription factor to regulate a different set of genes in each of these tissues (Halder and Carroll, 2001; Zhang et al., 2008). Because the SD protein can compete with the SD/VG complex for the same binding target sites, one is able to use the wing as a model system to gain some insight as to how SD is able to activate different sets of genes in a tissue specific manner. The data suggest that instead of changing the sequence that the SD protein recognizes, the VG protein alters the affinity the SD protein has for DNA. The reduced affinity for DNA is circumvented by the fact that the VG protein promotes the assembly of a complex that contains two SD proteins. These two features would ensure that the SD/VG complex could only bind to enhancers that contain two consecutive binding sites, a feature that is exclusively found in wing enhancers.

Previous studies have shown that the SD/VG tetramer on its own cannot induce the expression of downstream target genes (Halder and Carroll, 2001). Proper activation of these genes requires the SD/VG sites to be adjacent to signaling pathway transcriptional effector binding sites (Guss et al., 2001). The authors of this paper proposed that the signaling pathway transcription factors could be required to either help recruit the basal transcription machinery or to mediate chromatin remodeling. If the additional transcription factors are required to help recruit other cofactors then one should be able to bypass the requirement for these factors by replacing one of the VG terminal regions with a general VP16 activation domain. Our VP16/VG fusion constructs were unable to overcome the requirement for these factors. Because removing one of these activation domains prevents the complex from activating the *wg* downstream target gene, these

negative results do not completely exclude the possibility that the signaling pathway transcription factors are needed to recruit the basal transcription machinery. It could be possible that in addition to promoting the formation of the SD/VG tetramer and activating transcription, the terminal domains are required to perform another unknown task. It would be interesting to see if the fusion of a VP16 domain to a full length VG cDNA can induce the ectopic outgrowth of wing tissue in the eye.

The revised model explains how the VG protein promotes the specific activation of wing specific genes but does not address the question of how the cofactor Yorkie accomplishes this feat. The results herein do not exclude the possibility that Yorkie may alter the sequence that the SD DNA binding domain recognizes. The putative SD/Yorkie binding sites are much more conserved than the SD/VG binding sites. This increased conservation would be necessary if, indeed, the Yorkie cofactor modifies the sequence that the SD DNA binding domain recognizes. One way to test this hypothesis would be to perform electrophoretic mobility shifts assays (EMSA) with both the putative SD/Yorkie and SD/VG binding sites. If the Yorkie protein modifies the sequence that the SD protein recognizes, the SD/Yorkie complex should only be able to shift oligos containing the 5' NNGNATNT 3' sequence. Another possibility is that similar to the SD/VG story, the SD/Yorkie complex on its own cannot activate transcription. Proper induction of target genes could require other transcription factor complexes to associate next to the SD/Yorkie dimer. One could test this hypothesis by seeing if synthetic enhancers that contain only SD/Yorkie binding sites are able to induce the expression of a downstream reporter gene.

The work presented in this thesis raises several important questions. In chapter two, the data indicate that in addition to being required to properly develop the wing, the SD protein is also responsible for the proper development of the leg, the compound eye and the adult optic lobe. However, it is unclear as to why the loss of SD function leads to defects in these structures. At least in the case of the leg disc, it appeared that the SD protein is required for maintaining the survival of these cells. Work published after our findings confirmed that SD does play a general role in tissue survival and proliferation (Bandura and Edgar, 2008; Wu et al., 2008; Zhang et al., 2008). One important way to understand the role SD plays in the development of these tissues is to identify the specific cofactors SD is interacting with to pattern these structures. The previously identified roles of these cofactors could provide valuable insight towards deciphering the role SD plays in the formation of these structures. In chapter three, the Nerfin-1 transcription factor, was identified as a novel binding partner of SD. The characterization of the motifs required to facilitate this interaction revealed that the VG and Nerfin-1 cofactors use a domain of similar amino acid composition to interact with the SD protein. The functional significance of the SD/Nerfin-1 complex is still unclear. One key obstacle towards understanding the role of this complex is that the cells that co-express these two proteins are unknown. Cloning the enhancer of *sd* or developing an antibody towards the protein would allow one to identify these cells. Identifying these cells would allow one to genetically manipulate them to determine how the SD/Nerfin-1 complex facilitates their proper development. Finally, chapter four shows that enhancer selectivity in the wing requires the VG cofactor to decrease the affinity with which the SD protein binds DNA

and promotes the formation of a tetrameric structure. These two changes ensure that the SD/VG complex only binds to enhancers that contain two tandem TEAD binding sites. However it is unclear how enhancer selectivity is achieved with the SD/Yorkie or the SD/Nerfin-1 complex. Electrophoretic mobility shift assays with these two complexes would be a critical first step in understanding this problem.

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APPENDIX I

Primers Used to Make the Nerfin-1 Deletions

| | |
|---|---|
| Nerfin-1 fwd Start site | 5' GGTACCGGTACCatggcccagatacagacacc |
| Nefrin-1 fwd 163 | 5' GGTACCGGTACCATGcgatatctgaacctgaaaacc |
| Nerfin-1 fwd 361 | 5' GGTACCGGTACCATGccgcagagtccagcagggaag |
| Nerfin-1 fwd 547 | 5' GGTACCGGTACCATGcgcaataaagccacccgcaag |
| Nerfin-1 fwd 931 | 5' GGTACCGGTACCATGaatcgcaataccaccaaccag |
| Nerfin-1 fwd 1129 | 5' GGTACCGGTACCATGacaggcagttttcactttaac |
| Nerfin-1 rvs 3' end | 5' GAATTCCGAATTCCctcgatttcagtgggccagtg |

Each of the fwd primers were used with the Nerfin-1 rvs 3' end primer to make the Nerfin-1 deletion constructs. The sequence in capital letters are not part of the Nerfin-1 cDNA sequence

Primers Used to Make the VG and SD Constructs

SD fwd ATG Start

5' GGGGACAACTTTTCTATACAAAGTTGTAATGAAAAACATCACCAGCTCGAGC

SD rvs C terminal end

5' GGGGACAACTTTATTATACAAAGTTGTAAGCTTAAGCTTTTCCTTAATTAGACGG

VG fwd del N terminal ACT domain

5' GGGGACAACTTTTCTATACAAAGTTGTAATGCACACACACACGCATACGC

VG rvs del C terminal ACT domain

5' GGGGACAACTTTATTATACAAAGTTGTATTGCTGTTCCAGAACGACG

2XSID fwd

5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCACACACACACGCATACGC

2XSID rvs

5' ACAACTTTGTATAGAAAAGTTGGGTGATTGCTGTTCCAGAACGACG

5' ATG VP16 fwd

5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCCCCCCCGACCGATGTCAGC

5' ATG VP16 rvs

5' ACAACTTTGTATAGAAAAGTTGGGTGCCCACCGTACTCGTCAATTCC

3' VP16 fwd

5' GGGGACAACTTTGTATAATAAAGTTGTAGCCCCCCCGACCGATGTCAGC

3' STOP VP16 fwd

5' GGGGACAACTTTGTATAATAAAGTT GTATGAGCCCCCCCGACCGATGTCAGC

3' VP16 rvs

5' GGGGACCACTTTGTACAAGAAAGCTGGGTATGACCCACCGTACTCGTCAATTCC